Generation of Recombinant Designer Antibodies Using Phage Display for a Rapid and Subtype Specific InfluenzaDetection System

Von der Fakultät für Lebenswissenschaften

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meinen Kindern Annina und Etienne

Die Endlosigkeit des wissenschaftlichen Ringens sorgt unablässig dafür, dass dem forschenden Menschengeist seine beiden edelsten Antriebe erhalten bleiben und immer wieder von neuem angefacht werden, die Begeisterung und die Ehrfurcht.

Max Planck

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1 Summary

Influenza A/H5N1 viruses caused several panzootic outbreaks in the past. These viruses of avian origin have acquired a zoonotic potential by gene reassortment and mutations and can cross species barriers including humans. There is an urgent need of reliable broad spectrum H5N1 diagnostics. Therefore, the aim of this work is to identify H5 specific antibodies that can be applied in a subtype specific diagnostic system for the detection and discrimination of different influenza viruses.

In this work two single chain (sc)Fv antibody gene phage libraries were generated using chickens vaccinated and challenged with avian H5N1 influenza viruses from phylogenetically distant clades. For selection of antibodies two additional recombinant H5 antigens of human and avian origin were applied. Twelve unique anti-H5 scFvs were isolated from generated anti-H5 chicken immune phage libraries and were subsequently re-cloned into the bivalent IgG-like scFv-Fc antibody format for expression in mammalian cells. All of them showed binding to H5 hemagglutinin proteins in immunoblot assays and in ELISAs. No cross-reactivity with unrelated proteins was observed. The majority of selected binders detected a large variety of H5 viruses in immunoperoxidase monolayer assays (IPMA) and showed no cross-reactivity with other influenza viruses of closely related HA-subtypes H1, H2, H6 and H7. One antibody clone (JM7_8) showed very broad binding specificity for H5 ranging from LPAIV North American strains to HPAIV viruses of clade 2.2.1. Furthermore, neutralizing activity of those two antibody clones was demonstrated. Epitope mapping on peptide spot membranes revealed a six amino acid short epitope which is highly conserved among H5 influenza viruses. With a combination of scFv-Fc antibodies different recombinant H5 proteins as well as a selection of viruses of the H5 subtype were detected in sandwich ELISA and membrane based immunodot blot assay, demonstrating the proof of principle of a rapid diagnostic assay. Antibodies specifically stained influenza viruses of a purified avian low pathogenic H5 strain in transmission and scanning electron microscopy.

Thus, a successful immunization and selection strategy comprising four different H5 sources leading to the isolation of several H5 specific antibodies and elucidating a highly conserved linear epitope specific for H5 influenza viruses has been demonstrated. Isolated antibodies and the elucidated highly conserved linear epitope in the HA1 domain of the protein can be used in different applications e.g. influenza diagnostics, therapy and vaccination.

2 Zusammenfassung

Influenza A/H5N1-Viren haben in der Vergangenheit verschiedene Pandemien verursacht. Diese humanpathogenen Viren aviären Ursprungs haben durch Genaustäusche und Mutationen die Fähigkeit erworben, Artbarrieren zu überwinden. Für H5N1 besteht ein dringender Bedarf an Breitspektrum-Diagnostika. Derzeit ist das hochkonservierte Nukleoprotein das Zielprotein mit der weitesten Verbreitung bei Antikörper-basierten Diagnostika für Influenzaviren. Ziel dieser Arbeit war es daher, H5-spezifische Antikörper zu identifizieren, die in einem subtypspezfischen System zum Nachweis und zur Unterscheidung von Influenzaviren anderen Subtypes eingesetzt werden können.

Nach Vakzinierung von Hühnern mit aviären H5N1-Viren aus phylogenetisch entfernten „Clades“ wurden in dieser Arbeit zwei single-chain (scFv) Phage-Display Antikörperbibliotheken hergestellt. Zur Selektion der Antikörperfragmente wurden zwei zusätzliche rekombinante H5-Antigene verwendet. Aus den generierten anti-H5-Antikörperimmunbibliothekenaus immunisierten Hühnern wurden 12 unterschiedliche H5 spezifisch scFv-Fragmente isoliert und nachfolgend zur Säugerzell-Expression in das bivalente IgG-ähnliche scFv-Fc-Format umkloniert. Alle scFv-Fc-Antikörper binden beide H5-Antigene in Immunoblot- und in ELISA-Experimenten. Kreuzreaktivität mit weiteren Proteinen zeigte sich nicht.

Die Mehrzeit der selektierten Binder erkannte eine große Bandbreite an H5-Viren in Immunoperoxidase Monolayer Assays (IPMA) und zeigte keine Kreuzreaktivität mit anderen eng verwandten Influenzaviren der HA-Subtypen H1, H2, H6 und H7. Ein Antikörperklon (JM7_8) wies eine sehr breite Bindungsspezifität für H5. Darüber hinaus konnte neutralisierende Aktivität für zwei Antikörperklone gezeigt werden.

Die Epitop-Analyse auf membrangebundenen Peptiden führte zur Aufklärung eines 6-Aminosäure kurzen Epitops, welches innerhalb H5-Influenzaviren hoch konserviert ist.

Mit einer Kombination aus scFv-Fc-Antikörpern mit humanem bzw. murinem Fc-Fragment wurde für eine Auswahl an Viren ein H5-Sandwich-ELISA und ein membranbasierter Immuno-Dotblot-Assay durchgeführt. Hiermit konnte der Beweis erbracht werden, dass mit den isoierten Antikörpern der Aufbau eines schnellen diagnostischen Tests möglich ist. In Transmissions- und Rasterelektronenmikroskop konnte die spezifische Bindung der Antikörper an die Virusoberfläche gezeigt werden.

Damit konnte gezeigt werden, dass eine erfolgreiche Immunisierungs- und Selektionsstrategie zur Isolierung mehrerer H5-spezifischer Antikörper und zur Aufdeckung eines hoch konservierten linearen Epitopes führte, welches für H5-Influenzaviren spezifisch ist. Diese Antikörper und das Epitop können in verschiedensten Anwendungen eingesetzt werden.

3 Introduction

3.1 Influenza and influenza viruses

Three different types of influenza viruses can be distinguished according to the sequence of their nucleoproteins – influenza virus A, B and C. Although all three influenza types can infect humans, only influenza viruses of the types A and B cause remarkable morbidity and only influenza A viruses have the potential to cause pandemics due to their extraordinarily high antigenic variability[1].

Influenza A viruses are subgrouped according to antigenic characteristics of the two major viral surface proteins – hemagglutinin (HA) and neuraminidase (NA) and can infect a broad range of species, among them mammals, e.g. humans, horses, pigs, whales and seals (Fig 1). However, their natural hosts are wild water birds, where 16 of the known 17 hemagglutinin subtypes and all 9 neuraminidase subtypes have been isolated from.

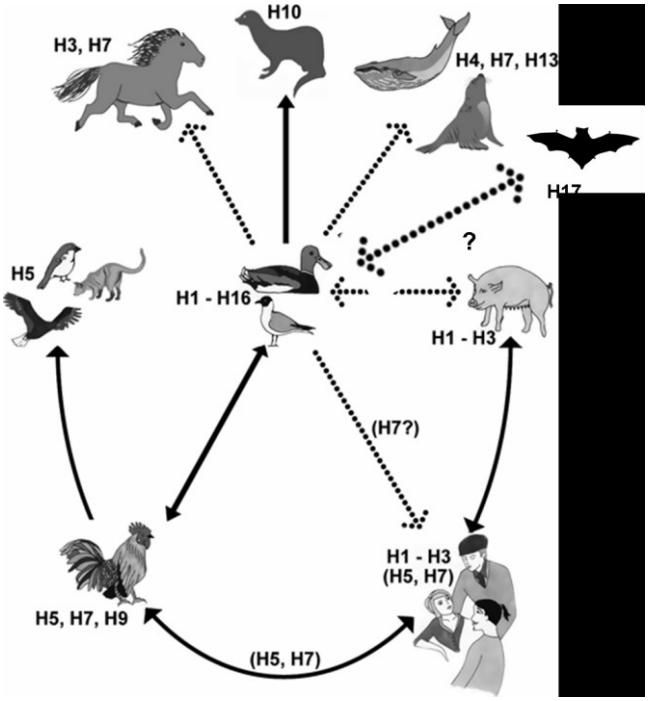

Figure 1: Host range of influenza A virus with the natural reservoir of influenza A virus.
Figure according to [2], modified

In their natural hosts, wild water birds, influenza virus infection often is restricted to the gastrointestinal tract and causes no or only mild symptoms, whereas in humans infection affects the upper respiratory tract with symptoms such as fever, chills, headache, sore throat, myalgia, and malaise [1]. At rare events highly pathogenic avian influenza viruses can develop which often have fatal consequences for the infected

host regardless of the species. Viral pathogenicity and transmissibility are critical in establishing a pandemic situation. Reasons for high pathogenicity are numerous and discussed in section 2.3. but a fatal result of influenza infection always seems to be a misrouted interplay of host organism and influenza virus without clear benefit for the pathogen unless high transmissibility supports virus spread.

Influenza A viruses of subtypes H1, H2 and H3 and neuraminidase subtypes N1 and N2 have circulated among the human population. Currently H1N1 and H3N2 as well as influenza B viruses are prevalent and can contribute to seasonal influenza outbreaks [3].

Since the beginning of the last century, humans were faced with four pandemics: in 1918/19 the so-called Spanish flu (H1N1) - the most devastating pandemic with more than 20 - 50 million deaths [1], Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968 and, most recently, the H1N1 pandemic in 2009.

3.1.1 Nomenclature of influenza viruses

Nomenclature of any influenza virus should follow a system established by WHO Expert Committee in 1980according to the genus (type), the species from which the virus was isolated (deleted if human), location of isolate (mostly country), the number of the strain (i.e. laboratory codes if any), the year of isolation followed by the HA and NA subtypes [4]. For example, an H5N1 influenza virus isolated from swan in Germany in 2006 would be A/whooper swan/Germany/R65/2006 (H5N1).

Due to the continuing evolution and diversification of H5N1 virus (Fig 2), a unified nomenclature has been suggested by the WHO[5]which is regularly updated according to the evolution of the virus. Hitherto, there are ten phylogenetic H5N1 clades designated 0 to 9 which are further divided into second, third and fourth order subclades. Currently, some orders of clade 1, 2 and 3 are circulating in Asia and Africa. For example, clade 2.1.3 in Indonesia, 2.2 in India and Bangladesh, 2.2.1 in Egypt (which further diversified in 2.2.1.1 clade and 2.2.1/C group) in addition to 2.3.2 and 2.3.4 are circulating in Asia. Figure (2) summarizes the recent diversity of clades 2.1 and 2.2.[5].

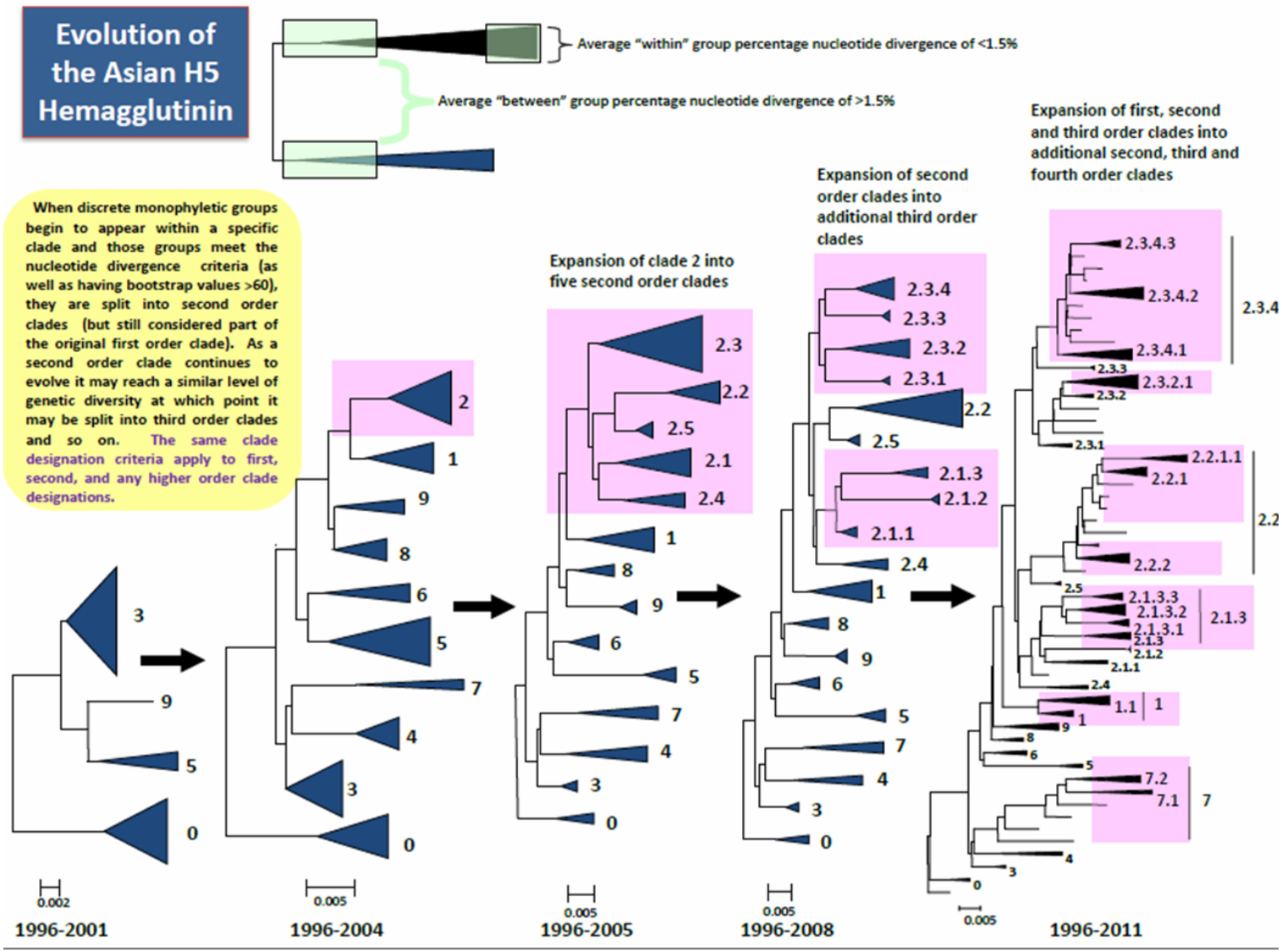

Figure 2: Conceptual diagram of ongoing H5N1 evolution until 27th May, 2012
according to [5]

3.1.2 Influenza virus and genome structure

Influenza viruses belong to the family of *Orthomyxoviridae*, are enveloped viruses of 80-120 nm in diameter and have a roughly spherical shape. The viral envelope contains two glycoproteins (HA and NA) and an ion channel protein (M2), all anchored in the lipid membrane of the virus. Viral RNA and its protecting and stabilizing proteins are organized in the central core of the virus. The M1 protein forms an inner circle protecting the viral ribonucleoprotein complex (vRNP). Influenza viruses possesseight segments (PB2, PB1, PA, HA, NP, NA, M and NS)of single stranded, negatively orientated RNA packed into ribonucleoprotein complexes (vRNP) encoding at least eleven viral proteins are encoded: the three surface proteins hemagglutinin (HA), neuraminidase (NA) and ion channel protein (M2), and the so-called internal poteins nucleoprotein (NP), M1 protein, NEP protein, non-structural proteins NS1 and PB1-F2, polymerase acidic protein (PA) and polymerase basic proteins PB1, PB2 [1] (Fig. 3). Each RNA segment consists of a coding segment flanked by a non-coding sequence at its 3' and 5' end of which the 12 – 15 terminal nucleotides are highly conserved among the eight RNA segments. Among all RNA viruses only influenza viral RNA is transcribed in the nucleus of the host cell and not in the cytoplasm [6]. The viral nucleoprotein is one of the most conserved proteins among all influenza viruses[7] whereas the two major surface proteins hemagglutinin and neuraminidase are highly variable between the different subtypes but, in the case of HA, also within the same subtype[8].

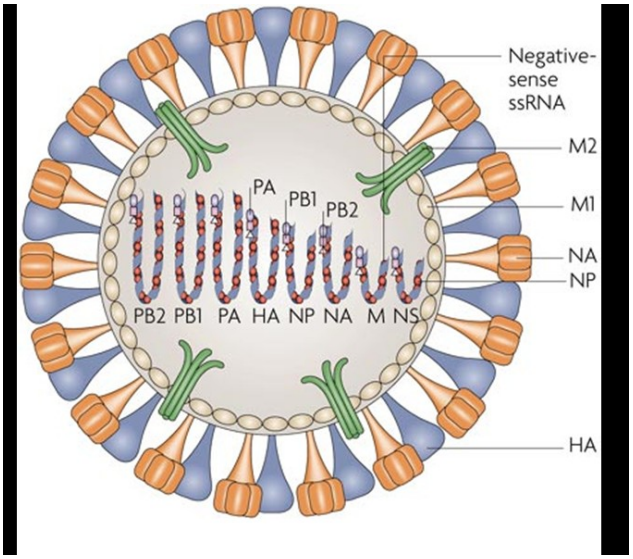

Figure 3: Schematic view of an Influenza virus
Viral surface proteins hemagglutinin (HA), neuraminidase (NA) and the ion channel protein (M2) are anchored in the viral membrane. The M1 protein foms an inner layer and binds the viral ribonucleic complexes (vRNPs) consisting of segmented viral RNA each associated with viral polymerase complex (not depicted) and the nucleoprotein (NP). Picture adapted from [9]

Using reverse genetics, a complete influenza virus can be constructed on the basis of cDNA [10] and this technique has been widely employed to investigate the contribution of each protein to the viral phenotype[6].

The influenza genome exhibits a huge variability and plasticity leading to continuous adaptation to the host, an efficient escape of host immune responses and the acquisition of properties that allow the virus to infect new hosts. Two major mechanisms contribute to the genetic variability – antigenic drift and antigenic shift. Whereas **antigenic drift** is described as a slow change in the antigens on the viral surface over time driven by the high error rate (1 misincorporated nucleotide per 10,000 nucleotides) of the viral RNA-dependent RNA-polymerase (vRdRp)[11], **antigenic shift** is a rapid change of antigens caused by a recombination of viral RNA segments in a host with concurrent infection of at least two different influenza viruses [12]. Such genetic rearrangements may give rise to viruses with pandemic potential since they can acquire the ability to infect new hosts or cause fatal infection due to a lacking immune response of the host.

3.1.3 Infection cycle of influenza viruses

The influenza virus infection process is initiated by the binding of viral hemagglutinin to host cell surface proteins terminated by an α-linked-N-acetylneuraminic acid residue ("sialic acid receptor")[13]. Following the attachment,the virus is endocytosed by a clathrin mediated pathway [14]and acidic milieuwithin the endosome leads to two effects on the virus –major conformational changes of the viral hemagglutinin and acidification of the inner viral space by proton influx through the M2-ion channel protein. Both processes in turn lead to the fusion of viral and endosomal membranes and to the release of viral RNA into the cytosol. Viral negative-stranded RNA segments are transported to the host cell nucleus, transcribed into mRNA and translated into viral proteins using host cell translation machinery. Transcription is associated with a cap-snatching mechanism resulting in chimeric mRNA-molecules possessing a polyadenylated host-derived cap that are transported into the cytosol and translated [15]. Replication and translation associated proteins re-enter the nucleus to be packed with viral genomic RNA replicated by the vRdRp to form RNPs which in turn, with the help of viral proteins M1 and NS2, are transported back to the cytoplasm. Progeny virions are released through a budding process and the sialidase activity of the viral neuraminidase that cleaves off

sialic acid moieties from the viral and host cell surface to prevent accumulation of virions at the cell surface. New influenza viruses are set free and can start a further infection cycle(Fig 4).

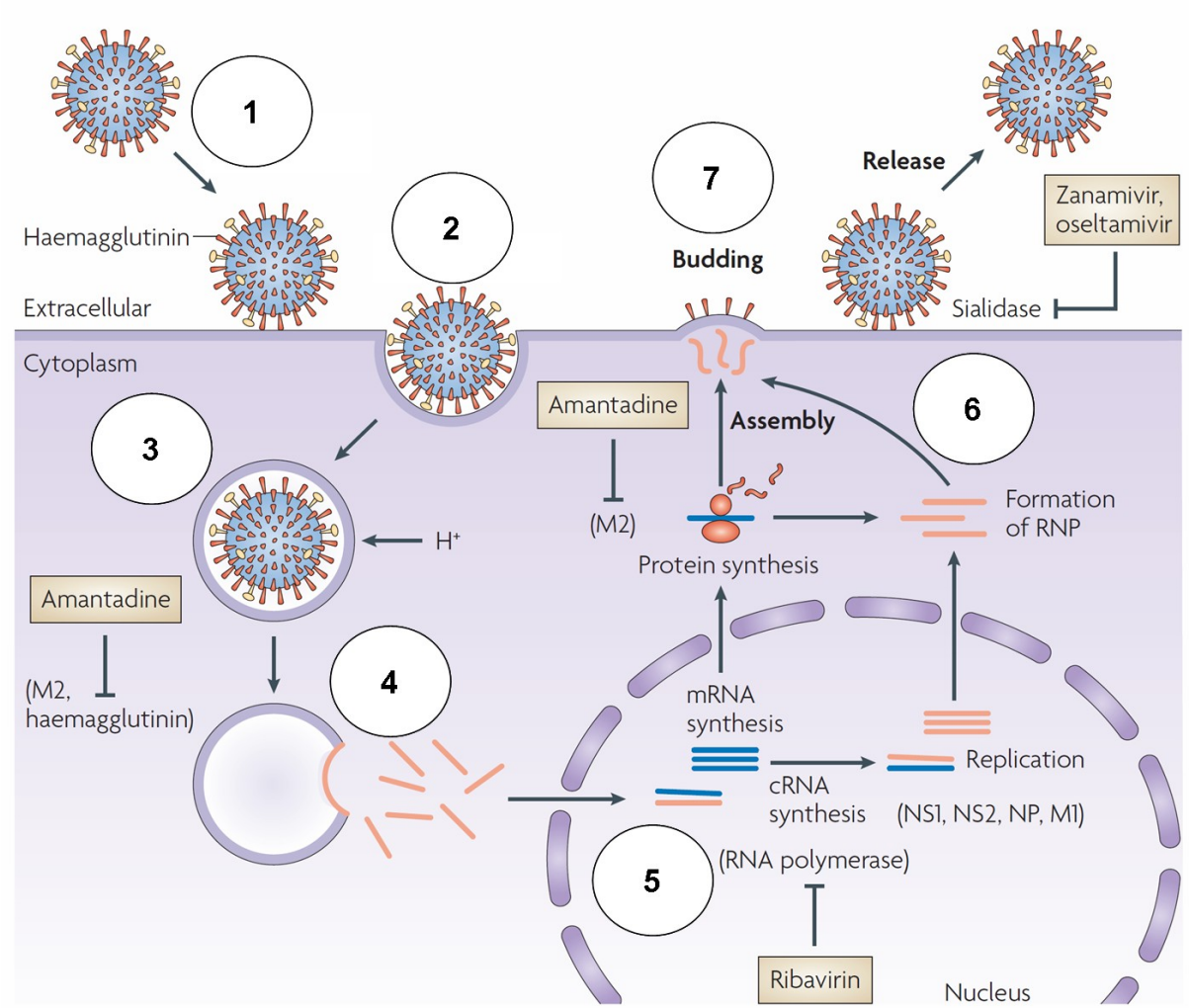

Figure 4: Life cycle of influenza virus and antiviral targets
1) attachment 2) endocytosis 3) endosomal acidification 4) uncoating of viral RNA and eclipse from endosome 5) replication and transcription of viral RNA in the host cell nucleus 6) virus assembly 7) budding of progeny viruses. Picture adapted from [16]

3.2 Influenza hemagglutinin

3.2.1 Structure of the influenza hemagglutinin

The hemagglutinin is one of three surface proteins of influenza viruses. It is a homotrimeric glycoproteinanchoring in the virus membrane with a transmembrane domain and can structurally be divided into a membrane distal globular head-like structure comprising a receptor binding site per monomere and most of the antigenicity determining epitopes, and a membrane anchored stem-like structure rich in α-helices[17]. A total of 17 subtypes are known. HA 1-16 were isolated from wild water birds, and only recently H17 was isolated from bats[18].The 16 subtypes exhibit a broad inter- and intra-

subtype variability. According to antigenic characteristics (ability to react with subtype specific hyperimmune sera [19,20] and amino acid sequence homologies (40 – 80 % sequence homology between different subtypes and ~ 90 % sequence identity within the same HA-subtype [21,22]) hemagglutinin subtypes are classified.

The hemagglutinin monomer is synthesised as an inactive precursor protein (HA0) which is proteolytically cleaved at a membrane near cleavage site for activation. Upon cleavage the hemagglutinin molecule is divided into two disulphide linked chains – HA1 with 328 amino acids in length and the HA2 stalk like structure with its 221 amino acids for H5[23].

Three main structural and functional characteristics of the influenza hemagglutinin have been elucidated: i) the receptor binding site at the tip of the globular head domain is responsible for host and cell specific attachment of the virus [24]; ii) at least four antigenic varible regions in the head-like part of the molecule help the virus to escape from host immune response [25]; iii) the cleavage site at the stalk-like membrane part of the molecule must be cleaved to completely acitvate the hemagglutinin, expose amino acids needed for the fusion of host and viral membranes to release viral RNA into host cell cytosol [24]. At least four antigenic sites have been determined [26] and are prone to amino acid variations to escape from host immune response.

The 17 HA subtypes discovered up to now can be divided into three groups as shown in Fig. 5; the H17 subtype only discovered recently has low sequence homologies with the longer known HAs and seems to represent an own group. The 16 remaining HA subtypes are grouped into five clades (H1a, H1b, H9, H3, H7) according to Medina & García-Sastre [27] and show three major structural differences i) in those regions of the hemagglutinin molecule that refold upon membrane fusion, ii) comprise a different orientation of the globular head region in relation to the central trimeric coiled-coil portion of the hemagglutinin molecule [28,29] and iii) cap forming residues of the HA2 subunit leading to the termination of the central trimeric coiled-coil structure [30,31].

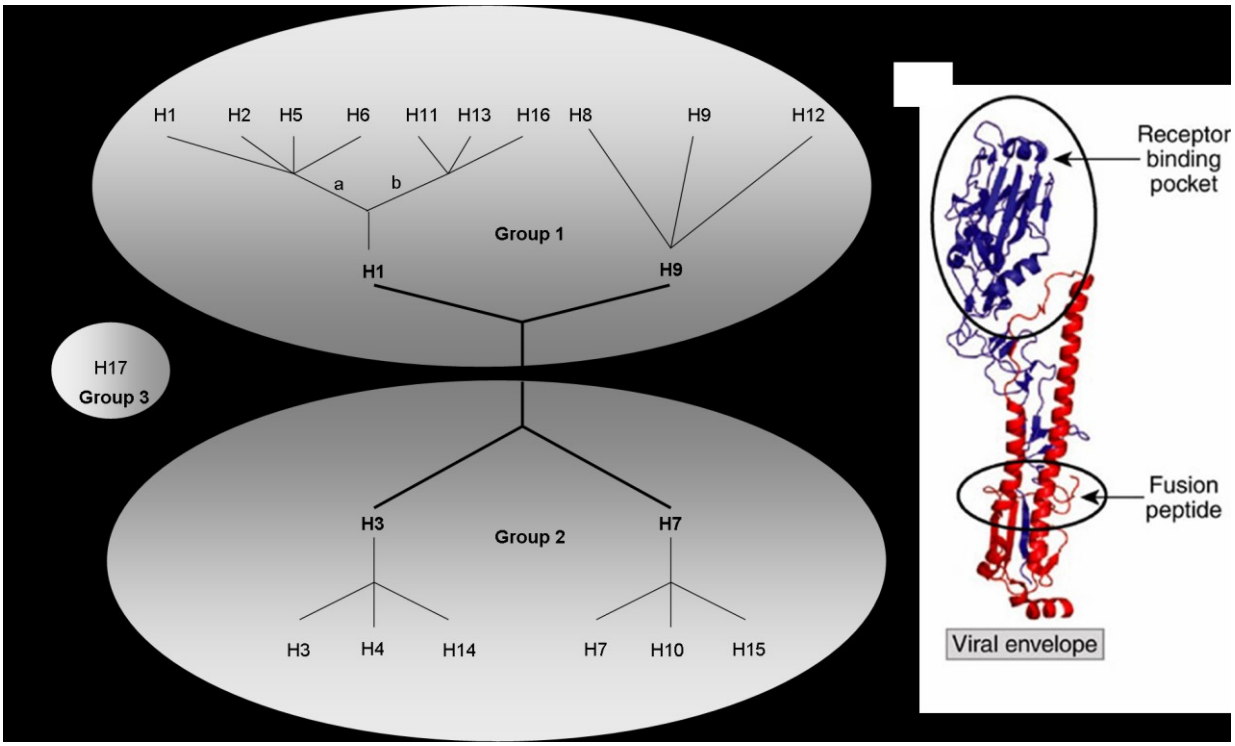

Figure 5: Classification and structure of influenza A virus hemagglutinin
a) Classification of influenza viruses into groups according to their antigenic properties is depicted. The two major groups are further subdivided into five clades(H1a, H1b, H9, H3, H7) and 16 subtypes; classification according to[27]. b) The monomeric structure of influenza hemagglutinin with its HA1 head-like structure (blue) comprising the antigenic sites and the receptor binding domain (shown by the arrow) and the HA2 stalk-like part (blue) with the cleavage site shown as fusion peptide is depicted. Picture adapted from [32]

3.2.2 Functions of the influenza hemagglutinin

Influenza hemagglutinin exhibits two major functions essential in the first step of virus infection: i) Attachment of the virus and ii) release of virus RNA from host endosomes into the cytoplasm after fusion of host endosomal and viral membranes.

Attachment of the virus

The binding affinity of influenza hemagglutinin to the host cell surface sialic acid receptor facilitates the attachment of the virus to host epithelial cells of the respiratory and gastro-intestinal tract – the major transmission route for influenza viruses [33]. Human and avian influenza viruses show major differences in their tissue tropism according to remarkably different host cell receptor recognition patterns. Avian influenza viruses preferably bind to sialic acid receptors linked to α2,6-galactose in glycosidic binding present on epithelial tissues of the gut and the lungs [34]. In contrast, the hemagglutinin of human influenza viruses has a higher affinity to α2,3-linked sialic acid receptors [35] predominantly present on non-ciliated human airway epithelial cells [36].

However, restriction of α2,3- linked receptors to human epithelial cells and α2,6-linked ones to avian cells is not exclusive as minor amounts ofα2,6-linked sialic acid can also be found on ciliated human epithelial cells and α2,3- linked receptors are present on chicken cells [37,38]. Pigs and quails express both types of galactose linkage to sialic acid receptors on their cells of the respiratory tract in sufficient amounts. Hence, they can more easily be infected with influenza viruses adapted to human and avian tissues. Pigs and quails are therefore regarded to be “mixing vessels” for influenza viruses as was demonstrated in the emergence of the H1N1 pandemic virus in 2009 [39–41]. Chickens also possess both receptor types – although preferentially in different organs and tissues [38].

Cleavage and membrane fusion

As the cleavage site of influenza hemagglutinin can either be very short (low pathogenic influenza viruses) or extended by an insertion of a variable amount of basic amino acids (highly pathogenic influenza viruses) the cleavage occurs either at the surface of the host cell after virus attachment by trypsin-like proteases or inside the cell by furin-like proteases or other proprotein convertases, respectively [42,43]. Cleavage and protonation of the hemagglutinin precursor lead to major conformational changes (Fig. 6).

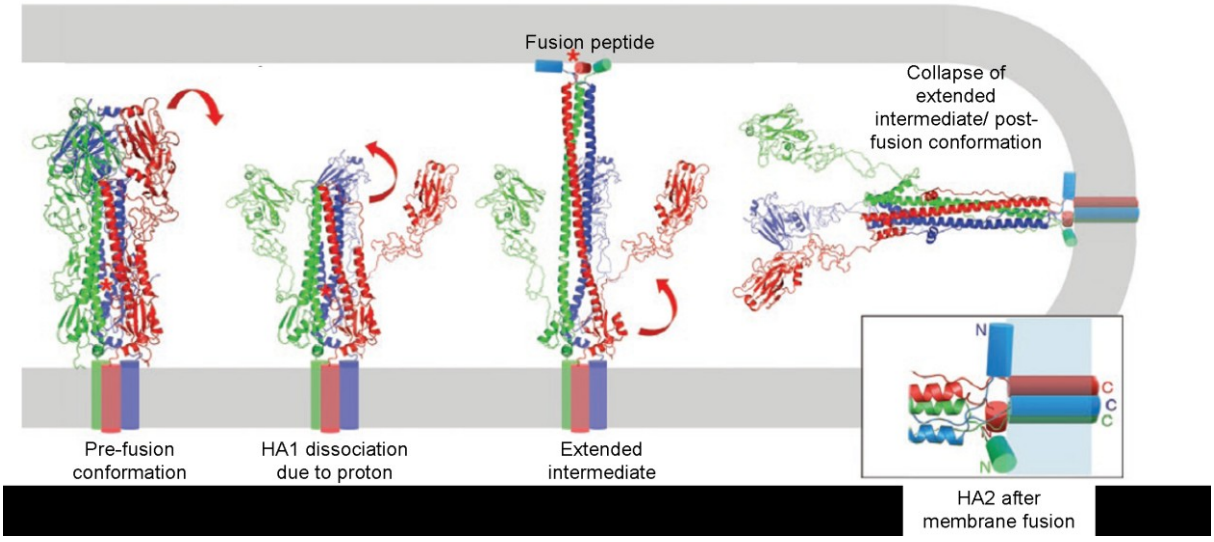

Figure 6: Conformational changes of the influenza hemagglutinin during the membrane fusion process
Figure according to[44]

3.3 Pathogenicity and virulence determining factors

The pathogenicity of influenza viruses is a multigenic issue and is considered to be the

result of different viral and host factors. In a plenty of studies it has been shown that mutations in many of the viral proteins including HA, NA, NS1, PB2, PB1-F2 contribute to viral adaptation to humans and that several host factors may facilitate infection of new hosts (e.g. humans) with influenza viruses (reviewed in [45,46]). Due to its functions in the first steps of infection the influenza hemagglutinin is suggested to play a major role in host restriction of influenza viruses [47–49]. Avian influenza viruses per se are not adapted to humans. After infection and replication the release of progeny viruses is the third process mainly driven by neuraminidase activity that needs host adaptation. Furthermore, intraspecies transmissibility of influenza viruses within a population of the new host must be guaranteed to establish an endemic situation. Tremendous efforts in elucidation of host-viral interplay have been taken during the last decades and the contribution of the changing nature of influenza viruses to the emergence of pandemic strains has recently been reviewed [50].

For all surface proteins, especially hemagglutinin and neuraminidase, glycosylation patterns seem to have an important impact on immunogenicity in the way that altered glycosylation positions may shield antigenic sites from host immune system and thus help to evade host immune responses and increase pathogenicity [51–53].

Certain antigenic sites of the **hemagglutinin** involved in the alteration of pathogenicity have been identified (Fig 7). The viral hemagglutinin shows adaptive mutations in its receptor binding sites from avian receptor to human receptors types. Mutations at positions 222, 224 as well as 182, 190 and 192 have been shown to be involved in receptor type switching[54]. Up to now, infections of humans with H5 avian influenza viruses have remained restricted to single cases with very close contact to infected birds. A recent study showed that four substitutions conferred respiratory droplet transmission in a ferret model for a reassortant consisting of the mutated H5 and the H1N12009 pandemic virus background [55]. Here, for the first time a transmission of a (yet lab-made) influenza virus with a highly pathogenic H5 hemagglutinin within a mammalian species has been demonstrated. Highly pathogenic H7N7 influenza viruses caused an outbreak in poultry in the Netherlands in 2003 that led to massive culling of poultry to restrict the transmission of the infection. For this virus it was demonstrated that an alanine to threonine substitution at position 143 of the hemagglutinin resulted in a change in location of infection from upper to lower respiratory tract by attachment to bronchial epithelial cells[56].

The cleavage site is another viral pathogenicity factor located in the viral surface protein hemagglutinin. In contrast to the single arginine representing the cleavage site of low pathogenic influenza viruses highly pathogenic variants show an insertion of basic amino acids of variable length. The amino acid composition of this cleavage site determines the localization of the infection as a single arginine cleavage site is cleaved by local trypsin-like proteases restricted to the respiratory and gastrointestinal tracts representing the site of first host-virus contact resulting in a localized infection[57,58].

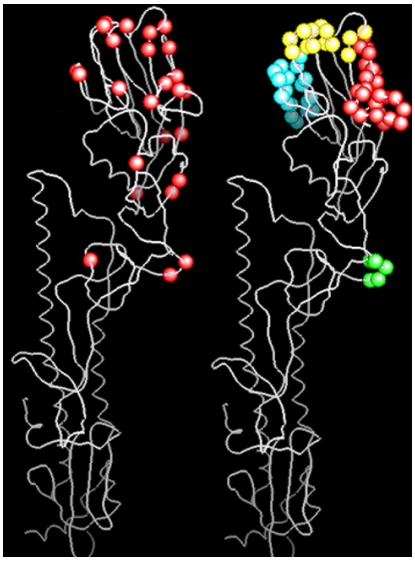

Figure 7: Comparison of positively selected vs antigenic sites of the influenza hemagglutinin H3
Crystal structure of a H3N2 virus was used according to[243]; left in red: amino acid positions with positive selection; right: the four antigenic regions A (red), B (yellow), C (green), D (blue). Picture adapted from[244]

In contrast multibasic cleavage sites require furin-like proteases that are ubiquitously distributed among different tissues, associated with the trans-Golgi-network of the cells[59]. Viruses with this type of cleavage site may provoke a systemic infection with often severe illness or fatal result. Glycosylation sires near the cleavage site can hinder or promote cleavage of he HA precursor protein by host proteases and thus contribute to the degree of pathogenicity[60].

Several other viral proteins contribute to pathogenicity. The length of the neuraminidase stalk region is variable depending on the virus isolate, yet a shorter stalk region results in a reduced acessibility of the substrates, sialic acids, seems to be associated with a decrease in virus release and hence reduced pathogenicity [61–63]. Nevertheless, highly pathogenic H5N1 avian viruses isolated from domestic poultry and wild birds in China (representing the so-called ”genotype *Z*” of H5N1 avian influenza viruses now endemic in southern China) as well as the H5N1 virus that caused the 1997 Hong Kong pandemic had a shortened stalk-region in line with obvious high pathogenicity[64]. In parallel to the hemagglutinin, amino acid substitutions in the neuraminidase have been shown to cause increased pathogenicity [65]. Other proteins like M2 (ion channel protein), different proteins of the viral polymerase complex and also NS1 are of clear importance in modifying pathogenicity [66–68].

3.4 Avian influenza viruses of the H5 subtype – outbreaks and lineages

Influenza viruses are wide spread among birds and hemagglutinin subtypes 1-16 can be found in the orders *Anseriformes* and *Charadriiformes*[8]. Grouping of influenza viruses into low and highly pathogenic avian influenza viruses (LPAIV and HPAIV, respectively) is based on viral pathogenicity measured in chickens. A small subset of influenza viruses has shown high pathogenicity in line with high mortality in domestic poultry as well as in wild birds and has proven to be able to cross species barriers and infect humans. In 1997, the first human fatal cases due to an infection with a highly pathogenic H5N1 virus were observed. This virus originated from sick geese in the chinese province Guangdong and was first isolated in 1996 [69]. A/Goose/Guangdong/1/1996 (H5N1) is regarded to be the origin of the ongoing panzootic evolution of H5N1 influenza viruses in Asian countries since 1997. Several outbreaks among industrial poultry flocks as well as among different wild birds [70,71] and re-current infections of humans underline the imminent threat to human health and industrial poultry production. Genetic and antigenic variability of the pool of circulating and already endemic highly pathogenic H5N1 viruses in Asia sheds light on its tremendous adaptive potential and the high risk for a new influenza pandemic. “Mixing vessels” like pigs and quails live in close contact to humans and poultry in those countries of ongoing H5N1 virus evolution and thus provide an excellent basis for further development, reassortment and spread, although low susceptibility of domestic pigs to HPAIV H5N1 was observed [72].

Based on the Guangdong virus isolate, the WHO proposes a hierarchical decimal numbering classification of clades and subclades of the emerged highly pathogenic H5N1 avian influenza viruses [73].

3.5 Tools and methods for detection of influenza viruses and influenza virus infection

Virus isolation and propagation is performed in embryonated chicken eggs (ECE) or in cell culture. ECE is the most common used system worldwide for obtaining replication-competent virus and considered to be the gold standard procedure for isolation and propagation of AIV[74]. However, it is laborious, expensive, time-consuming and increases the risk of biohazard contamination in case of HPAIV H5N1 [75–78]. Most commonly used cell cultures are Madin-Darby canine kidney (MDCK), primary chicken embryo kidney (CEK), primary chicken embryo fibroblast (CEF) cells [77]. Less often

used cell lines are duck fibroblasts, mink lung epithelial cells and quail fibroblasts[79]. Addition of trypsin to tissue cultures is pivotal for the isolation of LPAIV in contrast to HPAIV which are able to grow with or without additional trypsin [80].

Detection of influenza viruses is generally based on three different target groups: i) **viral antigens** (surface or internal proteins) , ii) **viral RNA** or iii) **antibodies** directed against different influenza proteins. To **detect viral antigens** (especially HA, NA matrix protein and NP)three different methods have been established and have been successfully applied: Lateral Flow Devices [75,81–83], Antigen-Capture Enzyme Linked Immunosorbent Assay (AC-ELISA) [84–86], Immunohistochemistry (IHC) [87–90]. Surveillance of influenza evolution and the rapid identification and subtyping of a potential pandemic influenza virus strain require fast, specific, sensitive and cheap techniques [91,92]. Recently developed **viral RNA detection**techniques involve the reverse transcription polymerase chain reaction (RT-PCR) [93–96], real-time RT-PCR (RT-qPCR) [75,91,97,98], Nucleic Acid Sequence Based Amplification (NASBA) [99–102], DNA microarrays [103–106] and sequence analyses [107,108] fundamentally contribute toAIV surveillance and characterization. **Antibody detection**plays a critical role in AIV surveillance and vaccine development[109]. Serological assays are inexpensive surveillance tools. Agar Gel Precipitation Test [96,110], Hemagglutination Inhibition and ELISA [96,111–114] are commonly used tests for AI antibody detection, with ELISA being the most sensitive test [75,84,115], whereas the HI test ismore specific in detecting AIV antibodies [116,117]. HI is a simple but laborious test based on the fact that antibody bound hemagglutinin (soluble or bound to the viral surface) fails to agglutinate as long as antibody binding interferes with the receptor binding activity of the hemagglutinin. HI is used in diagnosis and subtyping of AIV viruses (antigen and/or antibodies), with a panel of subtype-specific antisera or antigens/viruses representing all AIV subtypes to identify subtype-specific anti-AIV antibodies in different samples like serum, plasma, or egg yolk from different species [118]. Due to the variability of influenza hemagglutinin, the antigen used must be updated according to the field virus particularly for evaluation of post vaccination immune response [119–122].

3.6 Influenza therapy - Antivirals and influenza vaccines

Therapy of influenza virus infection in humans is based on antivirals that inhibit two different influenza virus proteins – the M2 ion channel protein and viral neuraminidase (Fig 4). In contrast to influenza specific antivirals Ribavirin inhibits RNA polymerases of

different RNA viruses.

3.6.1 Inhibitors of influenza ion channel protein M2

These M2 inhibitors are collectively referred to as adamantanes.**Amantadine** and **rimantidine**, first approved by the US FDA in 1967 and 1993, respectively, belong to this group of antivirals and block membrane bound viral ion channel protein M2 [123], thus preventing acidification of internal parts of the virus and release of viral DNA [124]. This inhibition occurs by binding of the hydrophobic adamantane drug to hydrophobic residues lining the pore in the outer regions of the M2 channel. Adamantanes are not active against M2 proteins of influenza B viruses because polar residues line M2 ion channel protein in the drug binding parts of the protein [125]. Consistent with these findings adamantane resistant escape mutants show polar amino acid residues at positions critical for drug binding instead of hydrophobic residues [126]. An increasing resistance of influenza A viruses to adamantanes limit the therapeutic potential of these antivirals. Already in 2005 the incidence of amatadine resistance was about 92 % in the US[127].

3.6.2 Neuraminidase inhibitors (NAI)

NAI reduce or abolish release of influenza viruses from infected cells by blocking the sialidase function of viral neuraminidase. Progeny viruses stay attached to cell surface sialic acid components and are not set free for a new infection cycle. These antivirals were designed to be structurally similar to host cell sialic acids and are active against influenza A and B viruses. Two different drugs group into NAI: oral drug **oseltamivir** (Tamiflu®, Roche) and inhaled drug **zanamivir** (Relenza®, GlaxoSmith-Kline), both approved by the US FDA in 1999. Both drugs have been demonstrated to be effective in prophylaxis and treatment of influenza infection [128,129], but increased resistance to NAI becomes a problem. In 2008 nearly 100 % of the seasonal H1N1 virus isolates were oseltamivir-resistant without a loss of virulence in these resistant isolates and resistance conferring mutation seems to be transmitted without selective pressure upon drug use since highest resistance levels have been found also in countries with low use of oseltamivir [130].

3.6.3 Vaccination

Vaccination programs in animals against highly and low pathogenic avian influenza viruses were initiated to prevent development of disease signs as well as to reduce mortality, decrease virus shedding into the environment, increase resistance of the host

to infection, limit bird-to-bird transmission and reduce losses in the egg production [131,132]. Furthermore, establishing a pre-immunized population to reduce the pandemic threat are main intentions of human seasonal influenza vaccination as well as animal HPAIV vaccination programs. Extensive vaccination programs have been shown to induce antigenic drift of influenza viruses [133,134];they may mask influenza infection of poultry flocks and interfere with serological surveillance[135–137]. Silent spread of influenza viruses in a vaccinated flock remains a problem since a 90 % protection of flock individuals due to vaccination only lead to a 50 % reduction in outbreak probability [138]. **Inactivated whole virus vaccines** and **recombinant vaccines**are the two main groups of influenza vaccines used[135]. **Inactivated homologous H5N1 vaccines**were genetically modified by reverse genetics to remove the multibasic cleavage site in the HA protein [139]. The major disadvantage of this strategy can be overcome by the use of **inactivated heterologous vaccines**that have the same HA subtype as the field virus but have a heterologous neuraminidase protein.Nevertheless, due to the antigenic drift of the virus afrequent update of the vaccine is required [120,140,141].**Recombinant vaccines**based on e.g.Fowl Poxvirus[142], Infectious Laryngotracheitis virus [143] or Newcastle Disease virus [144–146] have been used as vectorscarrying one or more influenza virus genes. However, antigenic matching of the vaccine to the currently circulating viruses is critical[147]. Vaccinated animals are discriminated from infected individuals by the detection of antibodies directed against influenza virus proteins not used in the recombinant vaccine virus and exclusively produced by influenza infected animals.

3.7 Antibodies

Immunoglobulins (Ig, antibodies) are the major players in humoral immune response of the adaptive immune system and are broadly applied in research, therapy and diagnostics.

Antibodies exert two different functions associated with the amino- and the carboxyterminal regions of the molecule. The highly variable aminoterminal region facilitates binding of the so-called antigen (viral or bacterial proteins, other molecules) and the highly constant carboxyterminal region of the antibody activates effector-mechanisms such as opsonizing and activation of the complement dependent cytotoxicity (CDC) and the antibody dependent cellular cytotoxicity (ADCC) [148]. Thus,

antibodies structurally link acceptor and effector function within a single molecule. Antibody response is initiated through antigen contact and human antibodies are built in the B-cells of the human lymphatic system.

The general structure of an antibody molecule described herein is based on human Ig molecules (Fig 8 a). The antibody consists of two identical heavy polypeptide chains (MW 50,000 Da each) and two identical light polypeptide chains (MW 25,000 Da each) forming a Y-shaped heterotetramer through disulphate bridges. The so called hinge region connects the two heavy chains to each other involving also additional covalent and non-covalent bonds. Two different classes of light chains are known: κ and λ, with the κ –chain being more dominant in the human antibody repertoire [149]. According to the constant regions of the heavy chains five isoforms of immunoglobulins (IgA, IgD, IgE, IgG, IgM) with different functions in antigen neutralization and opsonization and complement activation, can be distinguished. Depending on the isoform and the degree of multimerization antibodies have a molecular weight in the range of 150 (IgG) to 940 (IgM) kDa [150]. The chemical structure of antibodies was elucidated for the first time by Porter and Edelman in 1964 and both were awarded the Nobel Prize for this work in 1972. With about 80 % of the overall serum immunoglobulins affinity maturated IgG are the most abundant Igs in humans produced following an IgM-based primary response [148].

Antibody chains are composed of so-called immunoglobulin domains – each consisting of about 110 amino acid long repetitive sequences with four or two of them building up the heavy or light chain, respectively. Light chains consist of one variable and one constant domain, whereas heavy chains comprise one variable and three constant chains. The aminoterminal domain of each chain is characterized by a considerable high variabiliy with three hypervariable regions of 3-20 amino acids – the CDRs (*complementarity determining regions*) and the more or less constant framework regions flanking the CDRs [151,152]. The six CDRs of the heavy and the light chain form the antigenic binding site and each antibody posesses two of them conferring binding of the antigen through hydrogen bonds, Van-der-Waals forces and ionic bonds [148].

3.7.1 Antibody formats

Different antibody formats can be obtained from proteolytic cleavage with papain and pepsin and using recombinant methods. While pepsin cleaves the antibody in its hinge region leading to a bivalent $F(ab)_2$ fragment, papain activity divides the antibody into

two Fab-fragments (light chain and variable and first constant domain of the heavy chain covalently linked to each other) and the Fc-fragment [153].Recombinant antibody formats are widely used in diagnostics and therapy and have advantages e.g. smaller size, better tissue penetration, lack of complement activation due to the absence of the Fc-part [154–156] but also potential disadvantages as faster renal clearance and often – at least for scFv antibodies - reduced stability compared to full size immunoglobulins. Different recombinant antibody formats are used (Fig 8) with the scFv antibody representing the smallest antibody format consisting of the variable heavy and the variable light chain connected by a peptide linker, the Fab fragment with the variable and first constant domain of the light and the heavy chain covalently linked to each other and the bivalent scFv-Fc antibody with two scFv antibodies linked via a flexible region mimicking the hinge region to an Fc-fragment. This bivalent construct helps to overcome the frequently reported lower stability of single-chain scFv antibodies [157], increases avidity by dimerization of the scFvs, allows additional effector functions and facilitates detection via the Fc-part. Furthermore, by linking recombinant antibodies to effector molecules such as toxins, proteases, RNases or other drugs a site directed drug targeting can be achieved and is under broad investigation ([158,159]).

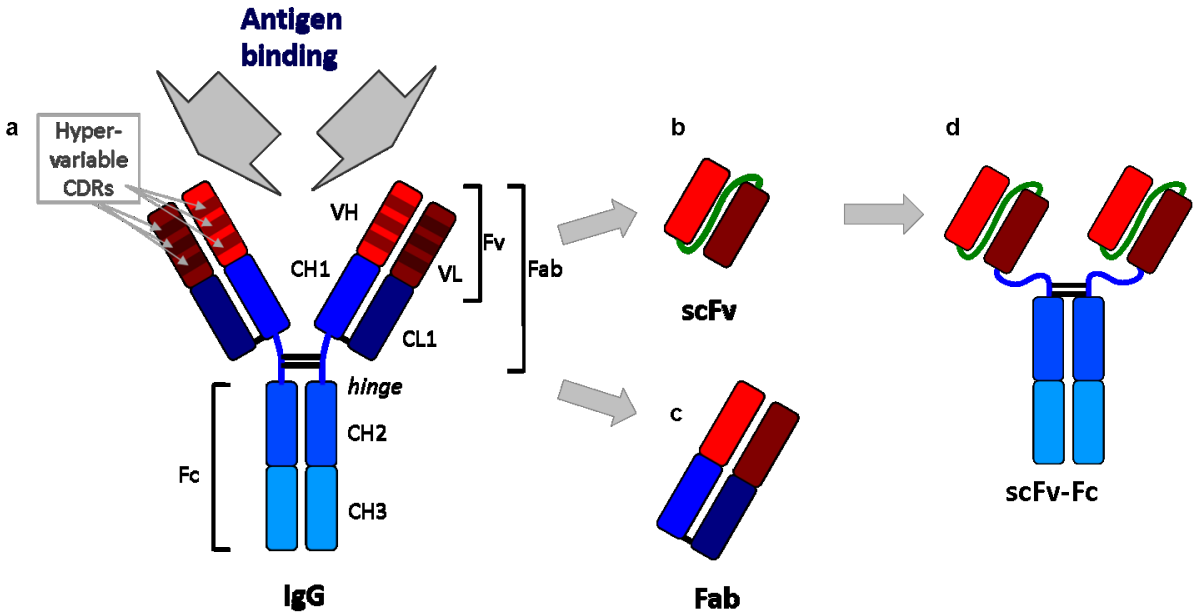

Figure 8: Antibody formats
a) Schematic view on the general structure of an immunoglobulin. The Y-shaped antibody consitsts of two heavy and two light chains that are connected by disulphate bonds in the hinge-region. The variable domains of both chains contribute to the antigen binding site.B) single-chain Fv antibody, c) Fab fragment, d) scFv-Fc antibody [154]

3.7.2 Chicken antibodies

Generally, chicken posses different immunoglobulin isotypes which are structurally and functionally related to human antibodies: IgM as the primary antibody response, IgA dominating immunoglobulins in body secretions and IgG (mostly referred to as IgY [160] due to structural differences compared to human IgG) representing the major isotype in serum and in egg yolk (reviewed in [161]). Passive immunization through maternal hen-derived IgY immunoglobulins in the egg yolk is a special characteristic of ovipar animals possessing IgY antibodies.

Beside the functional and structural similarities IgG and IgY (Fig 9) striking differences have to be mentioned as well: IgY antibodies possess four constant heavy (C_H1-C_H4) domains compared to the three C_H-domains of the human IgG,

and lack the hinge region which is not surprising and consistent with the hypothesis, that the hinge region is the result of a condensed C_H2-region [162]. IgY-antibodies show a decreased ability to precipitate antigens and exert notable skin sensetizing action that can mediate anaphylactic reactions [163,164] similar to human IgE. Disulfide bonding patterns are similar in IgY and primate IgE [165]. The chicken immunoglobulin repertoire is built in the bursa Fabricii, a mainly embryonic organ that starts to degenerate several weeks after hatching and is a remnant without function in 2-years old chickens [166].

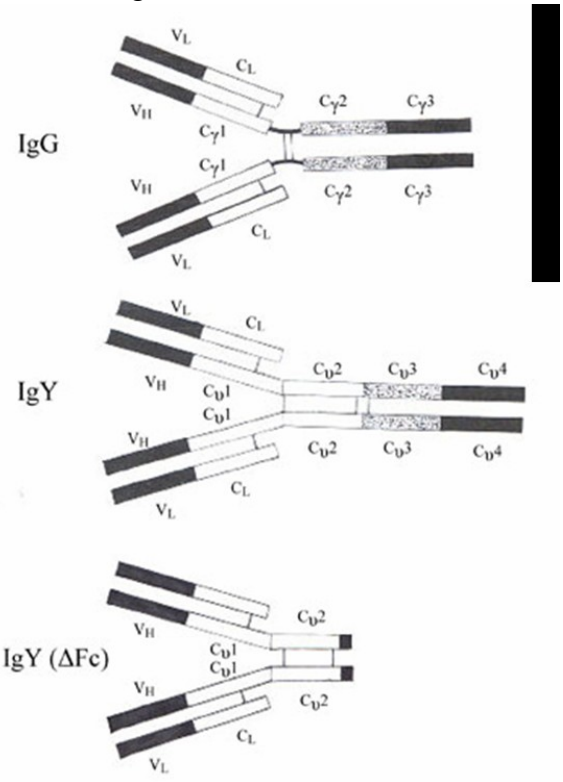

Figure 9: Structure of chicken IgY compared to human IgG antibodies.

The truncated IgY version present in some animals is shown as IgY(ΔFc)and possess only two C_H-domains, picture from[161]

In contrast to the diversity of V, J and D gene segments in the light and heavy chain locus known for humans, chickens have only one heavy chain (V_H) and one light chain (V_L) with only one functional V and J gene segment for each of them. Several pseudogenes (ψ) upstream of the V_H and the V_L gene segment (100 for V_H and 25 for V_L) - lacking recombination signal sequences and promoter region - serve as gene reservoir for the diversification of the chicken antibody repertoir by a process called

gene conversion [167–169]. CDR3 is encoded by the V-D-JH junction and its variability is further increased by a total of 15 D_H segments, and single base pair losses or additions at the D-segment during the process of gene conversion [168,170,171]. Since 100 pseudogenes contribute to V_H chain diversity compared to 25 pseudogenes for V_L chain, chicken antibody diversity is mainly heavy chain derived. Somatic hypermutations as known for human antibodies also contribute to affinity maturation of antibodies from the naïve immune repertoire of chickens the generation of which is completed several days after hatching. Reduced stability of chicken IgY under mild reducing conditions has been described[172] as well as a modified hydrolyzation of IgY by papain leading to F_{ab} fragments and degeneration of the Fc-fragment to dialyzable peptides [173].

3.8 Antibody phage display

The phage display technology allows for the selection of specific binders (either peptides or antibodies) to numerous antigens which are immobilized to a solid phase. These binders are genetically and physically linked to one of the surface proteins of the filamentous bacteriophage M13 and thus whole libraries of antigen binding molecules can be displayed on the phage surface and stored in a volume as small as a few microliters. A schematic view on a phage displaying an scFv antibody and on the genetic map of the antibody encoding phage display vector pHAL14 is provided in Fig 10. Compared to the hybridoma technology, another method to select for antigen specific antibodies, the selection process of phage display is performed completely *in vitro* with all possible advantages in designing the selection conditions such as adjusted pH-, salt- and temperature conditions, use of toxins as antigens and competitors during the selection process, duration of the selection, pre-selection on unfavourable antigens and implementation of multiple antigens during selection [174,175]. Furthermore, phage display is compatible with high throughput technologies thus allowing a rapid screen of numerous antigens or libraries [176]. An undisclosed freedom in the choice of the antigen (as long as it is soluble, immobilizable and of high purity) is the basis for the broad applicability of the phage display technology.

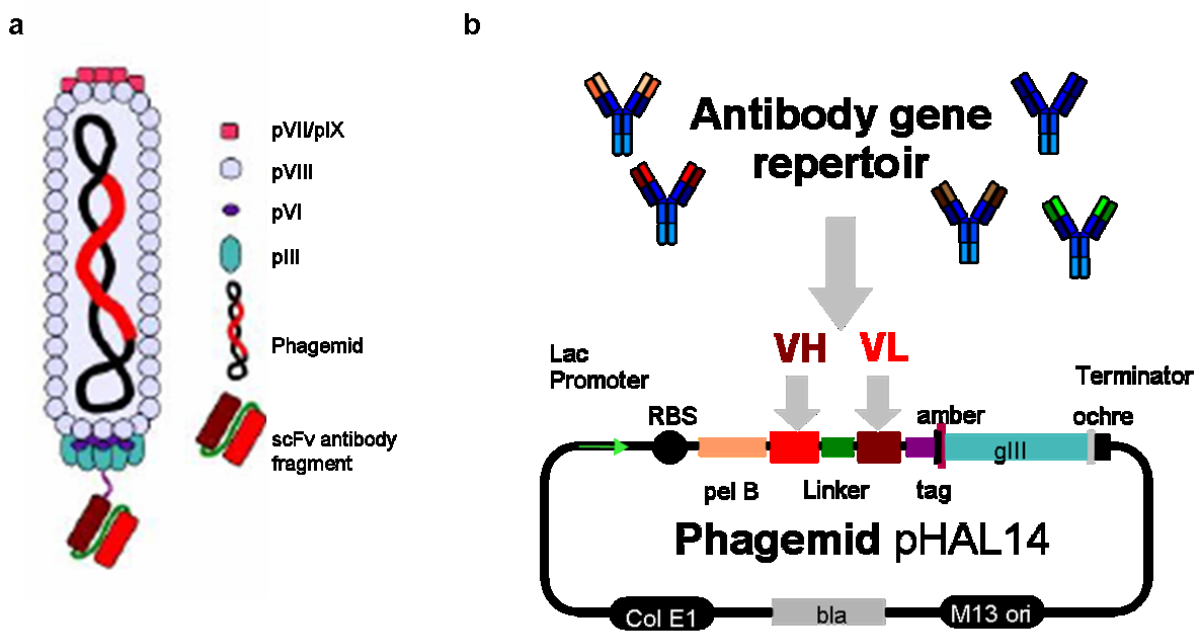

Figure 10: Schematic view on a phage displaying an scFv and the genetic map of the phage display vector pHAL14.
a) a phage presenting an scFv-antibody fused to pIII-protein, the five phage coat proteins pIII, pVI, pVII, pVIII and pX and, the phagemid DNA encoding the scFv sequence (red) and b) the phage display vector pHAL14 including an scFv; cloning sites, promoter (Lac Pr), ribosomal binding site (RBS), secretion leader peptide (pelB), ampicillin resistence gene (bla) and the two origins of replication (colE1 and M13 ori)[177].

Described for the first time in 1985 as peptide phage display with the genetic information for the peptide incorporated into the phage genome [178], phage display has overcome initial problems in the expression of antibody displaying phages and has become a very effective, successful and broadly used techniques in antibody isolation and selection. Using phagemids for encoding the antibody gene fused to the phage surface protein along with the *E. coli* origin of replication, marker genes, a multiple cloning site and the origin of replication of the filamentous phage allows the autonomous replication of the phagemid DNA within the host cell. A helper phage provides the genetic information for phage replication and wild-type phage proteins but is replication deficient due to a mutation in the helper phage gene II resulting in an increased packaging of phagemid DNA including the antibody-pIII-fusion. Thus, basically using phagemids for phage display, the antibody-pIII fusion product is more or less monovalently displayed on the phage surface with the wild-type pIII proteins derived from the helper phage. This monovalent display is of benefit for the selection of high affinity antibodies especially from immune libraries [179], whereas polyvalent display of antibodies with the help of a so-called hyperphage has been shown to be of advantage in the initial selection round when using naïve antibody libraries [180].All five phage coat proteins have been sucessfully used for phage display with pIII-display

representing the broadest applied method[181].

During the selection process – the so-called panning (Fig 11) –a solid phase immobilized antigen is incubated with the respective phage library follwowed by washing steps to remove unbound phages. After an elution step phage recovery is performed by infecting *E. coli* with eluted phages, following an amplification and purification of the phages from this first panning round. Within successive rounds of pannings stringency and panning conditions can be adapted according to the desired properties of the antibody to be selected.

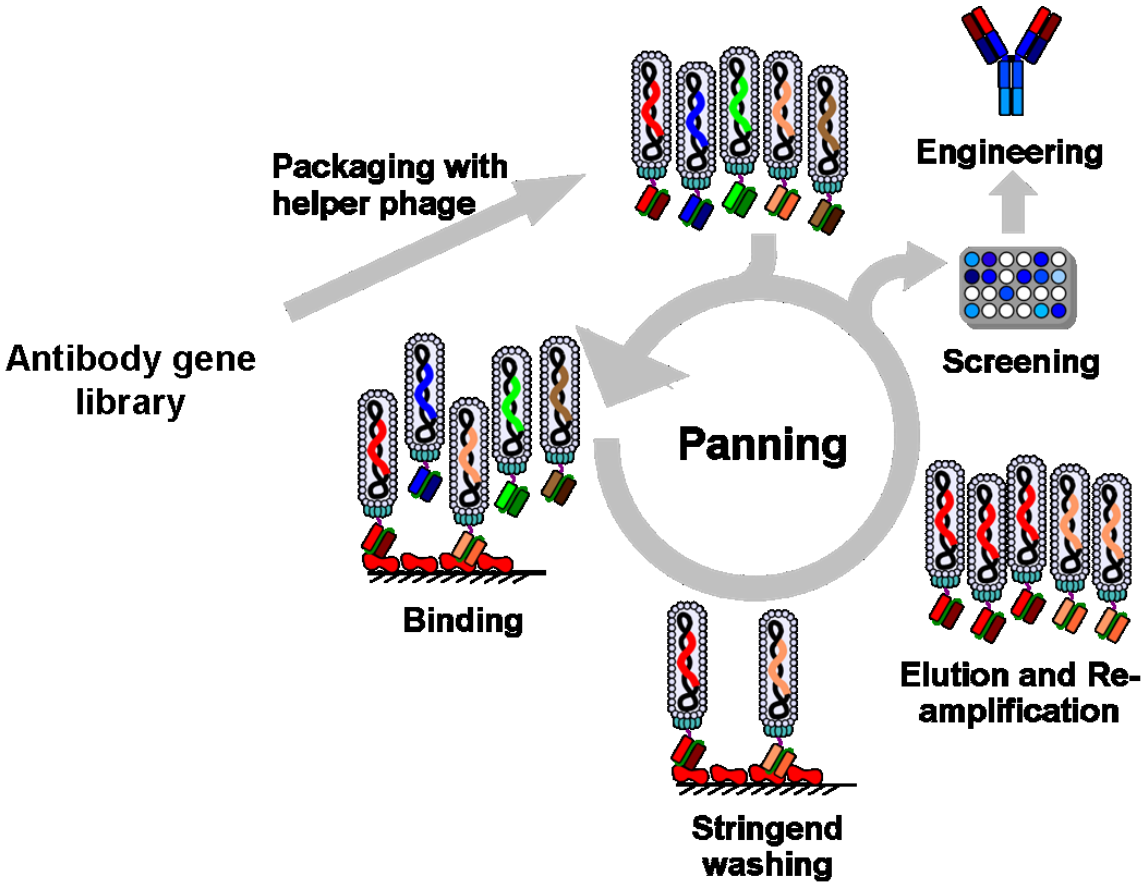

Figure 11: Selection cycle applied in antibody phage display (panning)
Figure adapted from[156]

3.9 Aim of the project

Despite all efforts influenza remains a constant threat to human health. Seasonal influenza causes more than 1 million deaths worldwide [182] and highly pathogenic influenza viruses have repeatedly caused devastating outbreaks in industrial poultry flocks. Moreover, formerly bird restricted H5N1 influenza viruses have acquired the property to cross species barriers and sporadically infect humans (WHO July 2012). Virus surveillance remains a demanding issue. Since up to now exclusively influenza viruses of the subtype H5, H7 have shown to develop into highly pathogenic influenza viruses and avian influenza viruses of the subtypes H5, H7 and H9 have infected humans, a subtype specific system for the detection and discrimination between these subtypes is highly desirable.

The aim of this work was to develop H5 specific antibodies toaddress the lack of antibody based subtype specific detection systems for H5 influenza. Chickens were immunized with phylogenetically different H5 influenza viruses and antibody gene libraries were constructed from chicken RNA. The constructed chicken anti-H5 immune antibody phage libraries as well as naïve human antibody phage libraries were used to isolate H5 specific antibodies. Two different recombinant H5 antigens from human and avian origin were implemented. Generated antibodies were analysed with regard to their binding specificity for H5 and other hemagglutinin subtypes, their neutralization and virus detection potential in several assays.

4 Material

4.1 Consumable material

Material	Supplier
Breathable sealing films for 96 well plates	Excel Scientific/ Sigma Aldrich, München
butvar-coated 300 mesh copper grids	Agar scientific, Essex, England
Culture dishes	
24 well cell culture, polystyrene	PAA Laboratories GmbH, Pasching
cell culture	Sarstedt, Nürnbrecht
Petri dishes	Greiner Bio-one, Frickenhausen
big plates	Sarstedt, Nürnbrecht
Dialysis membranes ViskingType 8/32 MWCO 14000	Carl Roth, Nürnbrecht
Electroporation cuvettes *Gene pulser* 0.1 cm	BioRad, München
Erlenmeyer flasks, polycarbonate with vented caps	Corning, Karlsruhe
Filter (celluloseacetate: 0.2 µm; 0.45 µm)	Sartorius, Göttingen
Filtertips (10 µL; 200 µL; 1000 µL)	Sarstedt, Nürnbrecht
Immunoblot filter	Schleicher-Schuell, Dassel
Kits	
PeqGOLD Plasmid Miniprep Kit I	peqLab, Erlangen
Nucleobond Xtra Midi	Macherey-Nagel, Düren
Nucleospin Extract II	Macherey-Nagel, Düren
Silver Staining Kit PlosOne™	GE Healthcare
SuperScript First-Strand Synthesis System for RT-PCR	Invitrogen, Karlsruhe
Magnetic beads	
Carboxylic beads M270	Invitrogen, Karsruhe
Epoxy beads M270	Invitrogen, Karsruhe
Streptavidin beads M280	Invitrogen, Karsruhe
mica strips SCD500	Bal-Tec, Liechtenstein

Material	Supplier
Micro-Schraubröhrchen	Sarstedt, Nürnbrecht
MTP Polypropylene, 96 well, flat bottom	Greiner Bio-one, Frickenhausen
MTP Polypropylene, 96 well, round bottom	Sarstedt, Nürnbrecht
MTP Polysyrole 96 well, Maxisorp	NUNC, Wiesbaden
MTP Polysyrole 96 well	NUNC, Wiesbaden
MTP polysorp, Streptavidin coated	NUNC, Wiesbaden
PVDF menbrane T830.1	Carl Roth, Nürnbrecht
Pipette tips (10µL; 200 µL; 1000 µL)	Sarstedt, Nürnbrecht
Reaction tubes 1.5 mL; 2 mL	Sarstedt, Nürnbrecht
Serological pipettes (2.5 mL, 5 mL, 10 mL, 25 mL)	Sarstedt, Nürnbrecht
Spatula sterile	VWR, Darmstadt
Syringes (2 mL, 5 mL)	B. Braun; Melsungen
Syringe Filter, single use, Minisart RC4, 0.45 nm	Sartorius, Göttingen
Tubes polypropylene 15 mL, 50 mL	Corning, Karlsruhe

4.2 Equipment

Equipment	Description	Supplier
Blotting Equipment	TransBlot Semi DryTransfer Cell	BioRad, München
	Multiscreen Mini Protean III	BioRad, München
Protein Purification System	Profinia	BioRad, München
Electrophoresis System	Biotech. Model 40-0708/40-314	peqlab Biotech GmbH, Erlangen
	Mini Protean III	BioRad, München
Electroporator	Micropulser	BioRad, München
ELISA System	Reader TECAN Sunrise	TECAN, Crailsheim
	Washer Columbus pro-basic	TECAN, Crailsheim
	TECAN Hydroflex Bead washer	TECAN, Crailsheim
Filter system	Bottle top	Nalgene, Sartorius

Equipment	Description	Supplier
Gel documentation	Gel documentation	Intas, Göttingen
Incubators	BE400	Memmert, Schwabach
	Thermomixer 5436	Eppendorf, Hamburg
	Hera Cell Heraeus (with CO_2-supply)	Ehret GmbH, Emmendingen
Laminar flow boxes	Thermo Scheintific Msc-Adventage	
	Heraeus, Herasafe	
Magnetic bead separator	Dynal-MPC	Invitrogen, Karlsruhe
PCR Cycler		
Photometer	Nanodrop ND-1000	peqlab Biotech GmbH, Erlangen
	Biochrom LTB Libra S11	Biochrom, Berlin
Shakers	Multi-Shaker Rotator RS-24	G Kisker
	Inforr HT Multitron	Infors AG, Bottmingen, Schweiz
	Certomat BS-1	B. Braun Biotech, Melsungen
	Infors HT Minitron (with CO_2 supply)	Infors AG, Bottmingen, Schweiz
	MTP-shaker Thermoshaker PST 60 HL-4	Lab 4you, Berlin
Scales	Sartorius excellence 1203 MP	Sartorius, Göttingen
	EMB 220-1	Kern, Balingen-Frommern
Vortex		
Centrifuges	Eppendorf 5810R	Eppendorf, Hamburg
	Eppendorf 5415D	Eppendorf, Hamburg
	Sorvall RC5B Plus	Kendro, Hanau
	Sorvall RC6 Plus	Kendro, Hanau
	Heraeus Multifuge 3 S-R	
Electron microscopy	910 TEM	Zeiss, Oberkochen, Germany
	DSM982 Gemini field emission scanning electron microscope	Zeiss, Oberkochen, Germany
	In-lens SE-Detektor	Zeiss, Oberkochen, Germany

Equipment	Description	Supplier
	Everhart-Thornley SE-detector	Zeiss, Oberkochen, Germany
	Slow-Scan CCD-Camera (ProScan, 1024x1024)	Scheuring, Germany

4.3 Chemicals

Used chemicals were supplied, except as noted otherwise, by Fermentas (St. Leon Rot), Merck (Darmstdt), Roche (Mannheim), Carl Roth (Karlsruhe), Sigma Aldrich (Fluka, Riedel de-Haen) and had p.a. quality.

4.4 Buffers, media and solutions

All buffer, media and solutions were prepared, except as noted otherwise, in ultrapure water. Media were autoclaved prior use and thermolabile components were filtered and added under sterile conditions.

Declaration	Components	Concentration
General buffers and solutions		
PBS	NaCl	145 mM
	$NaH_2PO_4xH_2O$	7.5 mM
	$Na_2HPO_4xH_2O$	2.5 mM, pH 7.4
Gycerol	Glycerol	80% (v/v)
Tween 20	Tween 20	0.05% (v/v)
Affinity chromatography Protein A (Profinia), working formulations		
Binding buffer/Desalting buffer	NaCl	137 mM
pH 7.0	KCl	2.4 mM
	Na_2HPO_4	4.3 mM
	KH_2PO_4	8.5 mM
Binding buffer (high salt)	NaCl	2 M
	KCl	2.4 mM
	Na_2HPO_4	4.3 mM
	KH_2PO_4	8.5 mM
Elution buffer pH 3.0	Citric acid	100 mM
	Sodium acetate	100 mM
Cleaning buffer 1, pH 8.0	NaCl	500 mM
	Tris	50 mM

Declaration	Components	Concentration
Cleaning buffer 2, pH 4.4	NaCl	500 mM
	Sodiumacetate	100 mM
Storage buffer	Benzylalcohol	2 % (v/v)
CIP buffer affinity column	NaOH	0.1 M
Agarose gelelectrophoresis		
Agarose	agarose	0.8-1.5% (w/v)
Ethidiumbromide solution		
Loading buffers		
X-buffer 6x	Tris-HCl pH 7.6	10 mM
	xylene cyanol FF	0.03% (w/v)
	glycerol	60% (v/v)
	EDTA	60mM
B-buffer 6x	Tris-HCl pH 7.6	10 mM
	bromophenol blue, 0.03%	0.03% (w/v)
	glycerol	60% (v/v)
	EDTA	60mM
Running buffer TAE pH 8.0	Tris HCL	4 mM
	Acetic acid	2 mM
	EDTA	1 mM
Coomassie Staining		
Staining solution	Comassie Brilliant blue GLR-250	0.05% (w/v)
	Acetic acid	10% (v/v)
	2-propanol	25% (v/v)
Destaining solution	Acetic acid	10% (v/v)
Coupling to magnetic beads		
Carboxylic beads		
NHS	NHS	0.1 M
EDC	EDC	0.5 M
Sodium acetate solution pH 5.0	sodium acetate	10 mM

Declaration	Components	Concentration
Ethanolamine-HCL pH 8.5	Ethanolamine-HCL	50 mM
Epoxy beads		
PBS, pH 7.4	PBS	
Borate buffer pH 8.6	sodium borate	100 mM
Streptavidin beads		
PBS, pH 7.4	PBS	
Cultivation of bacteria		
dYT culture medium	Bacto-yeast extract	1.0 % (w/v)
	Bacto-tryptone	1.6 % (w/v)
	NaCl	0.05 % (w/v)
dYT agar	Bacto-Agar in dYT culture medium	1.5 % (w/v)
dYT-GA	dYT culture medium	
	Glucose	100 mM
	Ampicillin	100 mg/L
dYT-T	dYT culture medium	
	Tetracyclin	50 mg/ L
dYT-GAT agar	Bacto-Agar in dYT culture medium	1.5 % (w/v)
Glucose solution	Glucose	2 M
IPTG solution	IPTG	1 M
Mg^{2+} solution I	$MgCl_2$	1 M
Mg^{2+} solution II	$MgSO_4$	1 M
SOC	Bacto-Tryptone	2.0 % (w/v)
	Yeast extract	0.5 %
	NaCl	0.005 % (w/v)
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM

Declaration	Components	Concentration
ELISA		
Blocking solution milk	PBS	
	Tween 20	0.05 % (v/v)
	milk powder	1-2 % (w/v)
Blocking solution BSA	PBS	
	Tween 20	0.05 % (v/v)
	BSA	1-2 % (w/v)
Washing buffer	PBS	
	Tween 20	0.05%
Immobilization buffer pH 9.6	sodium carbonate	50 mM
Declaration	**Components**	**Concentration**
Developing solution	TMB solution A	95 % (v/v)
	TMB solution B	5 % (v/v)
Stopp solution	H_2SO_4	1N
Epitope mapping		
T-BST buffer		
CBS buffer		
Developing solution	CBS buffer	
	$MgCl_2$	5 mM
	BCIP	0.006 % (w/v)
	MTT	30 µg/ mL
Stripping buffer A pH 7.0	Urea	8 M
	SDS	1 % (w/v)
	β-Mercaptoethanol	0,5 % (v/v)
Stripping buffer B	10 % Essigsäure, 50 % EtOH in H_2O	
MTT solution	MTT	5 mg/ mL
Expression of scFv-Fc antibodies in eukaryontic cells		
Transfection solution	PEI	
	DMEM	

Declaration	Components	Concentration
DMEM (High glucose)	FCS	8 %
	Penicillin/Streptomycin	1 % (v/v)
Penicillin/Streptomycin	Penicillin/Streptomycin (100x)	1 M
F17		
F17 complete	FreeStyle F17	
	glutamine	7.5 mM
	pluronic F68	0.1 % (w/v)
Immunoblot		
Blocking solution	PBS	
	Tween 20	2 % (v/v)
Washing solution	PBS	
	Tween 20	0.05 % (v/v)
Incubation solution	PBS	
	Tween 20	0.05 % (v/v)
	milk powder	1 % (w/v)
Transfer buffer	Glycine	192 mM
	Tris HCl	25 mM
Developing solutions AP conjugate		
BCIP solution	Dimethylformamide	100 % (v/v)
	BCIP	1.5 % (w/v)
NBT solution	Dimethylformamide	70 % (v/v)
	NBT	3 % (w/v)
AP-Substrate buffer pH 9.5	Tris HCL	100 mM
	$MgCl_2$	0.5 mM
Developing solutions HRP conjugate		
DAB solution	DAB	25 mg/ mL (w/v)
	PBS	
	$CoCl_2$	0.0 2% (w/v)
H_2O_2 solution	H_2O_2	37 % (v/v)

Declaration	Components	Concentration
HRP-Substrate buffer	DAB	2.5 mg/ mL (w/v)
	H_2O_2	0.0037 % (v/v)
IPMA		
Fixation solution	methanol	40 % (v/v)
	acetone	60 % (v/v)
Wash buffer	TBS	
	Tween 20	0.05% (v/v)
Staining solution	DMF	2.5 mL (100 %)
	AEC	1 pellet
Phage preparation buffers		
Phage Precipitation buffer	PEG 6000	20 % (w/v)
	NaCl	2.5 M
Phage dilution buffer, pH 7.5	Tris HCl	10 mM
	NaCl	20 mM
	EDTA	2 mM
PCR		
dNTPs	dNTP (Fermentas)	10 mM
Purification of influenza viruses		
Sucrose gradient I	sucrose	60 % (w/v)
		35 % (w/v)
Tris-buffer	Tris pH 7.4	100 mM
	EDTA pH 7.2	3 mM
Sucrose gradient II	sucrose	60 % (w/v)
		50 % (w/v)
		40 % (w/v)
		30 % (w/v)
SDS-PAGE		
Acrylamide solution	Acrylamide	30 % (w/v)
	Bisacrylamide	0.8 % (w/v)
APS solution	APS	10 % (w/v)

Declaration	Components	Concentration
Loading buffer 5x (Laemmli)	TrisHCl pH6.8	0.312 M
	SDS	10 % (w/v)
	Glycerin	50 % (v/v)
	β-Mercaptoethanol	15 % (v/v)
	Bromophenolblue	0,02 %(w/v)
Running buffer	Glycine	192 mM
	SDS	0.1 % (w/v)
	Tris HCl	25 mM
SDS solution	SDS	10 % (w/v)
Tris HCl-solution pH 6.8	Tris HCl	1.5 M
Tris HCl-solution pH 8.8	Tris HCl	1 M

4.5 Bacterial strains, cell lines and viruses

Bacterial strain	Name	Properties / Cultivation	Supplier
E. coli	XL1-*Blue* MRF'	*supE44 hsdR17 recA1 endA1 gyr A46 thi relA1lac-F' [proAB+lac^{g} ZDM15 TN10 (tet^{R})*	Agilent Technologies Deutschland GmbH, Böblingen
Cell Line	**Name**	**Properties / Cultivation**	**Supplier/Reference**
HEK 293T	ATCC CRL-11268	human embryonic kidney cells, adherent	Sena-Esteves *et al*, 1999
HEK 293-6E		human embryonic kidney cells, growth in shaking culture[183]	

All influenza viruses were supplied by the FLI and exclusively used in the laboratories of the FLI. All influenza viruses were supplied as allantoic fluid supernatant produced in embryonated chicken eggs and were not inactivated.

H/N nomenclature	Pathotype	Virus strain	Supplier
H5N1	HP	A/Vietnam/1197/2004 (NiBrg14)	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N1	LP	A/teal/Föhr/Wv632/05	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N2	LP	A/goose/Manitoba/428/06	FLI, Isle of Riems, Germany, Prof. Dr. Harder

H/N nomenclature	Pathotype	Virus strain	Supplier
H5N1	HP	A/Whopper swan/R65/DE06	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N2	LP	A/duck/Potsdam/1402/86	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N2	LP	A/mallard/Ger/Wv476/04	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N3	LP	A/mallard/Ger/Wv1349/03	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N6	LP	A/duck/Potsdam/2216/84	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N9	LP	A/chicken/Italy/22/98	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5N9	LP	A/mallard/B.C./544/05	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H1N1	LP	A/duck/Germersheim/R30/06	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H2N2	LP	A/guinea fowl/Ger/D23/85	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H6N2	LP	A/turkey/Germany/617/07	FLI, Isle of Riems, Germany, Prof. Dr. Harder

4.6 Recombinant hemagglutinins

Name	Origin	Expression system	Supplier
H5 A/Anhui/012005(H5N1, HP)	human	insect cells	Sino Biologicals GmbH, China
H5 A/Canada goose/Germany/71/06[184] (H5N1, HP)	avian	insect cells	FLI, Isle of Riems, Germany, Prof. Dr. Harder
H5 A/chicken/Egypt/0879-NLQP/2008-R737/09 (H5N1 HP)	avian	insect cells	FLI, Isle of Riems, Germany, Prof. Dr. Harder

H7 A/chicken/Germany/R28/03[184] (H7N7, HP)	avian	insect cells	FLI, Isle of Riems, Germany, Prof. Dr. Harder

4.7 Plasmides

Plasmid	Application
pHAL 14	phagemid –vector, construction of antibody-libraries, phage-display
pCMX2.5-hIgG1Fc-XP	eucaryontic expression vector, expression of scFv-humanIgG1Fc-antibodies
pCMV2.5-mIgG1Fc-XP	eucaryontic expression vector, expression of scFv-mouseIgG1Fc-antibodies
pCSE2.5-mIgG2cFc-XP	eucaryontic expression vector, expression of scFv-mouseIgG2cFc-antibodies

4.8 Oligonucleotides

Oligonucleotide	Sequence 5'-3'	Application
MHChick VH_f	gccgtgacgttggacgagtcc	
MHChick VH-r2	ggttgcactcccggaggagaccatgacttcggtcc	
MHChick VL-f	gcgctgactcagccgtcctcg	
MHChick VL-r	acctaggacggtcagggttgtc	
MHChick VH_*Nco*I_f	gtcctcgca*ccatgg*ccgccggacgttggacgagtcc	
MHChick VH_*Hind*III_r2	gtcctcgca*aagctt*tggggcggatgcactcccggaggagac	
MHChick VL_*Mlu*I_f	accgcctcca*acgcgt*agcgctgactcagccgtcctcg	
MHChick VL_*Not*I_r	accgcctcc*gcggccgc*acctaggacggtcagggttgtc	
MhlacZpro_f	ggctcgtatgttgtgtgg	
MhgIII_r	ctaaagttttgtcgtctttcc	
Tor-pCMV-mIgG01-FC-seq-f	cactttgcctttctctcc	Amplification of scFv pCMV2.5-mIgG1Fc pCMX2.5-hIgG1Fc pCSE2.5-mIgG2cFc
Tor-pCMV-mIgG01-Fc-seq-r	cagatggctggcaactag	
random hexamer oligonucleotides		cDNA synthesis

Oligonucleotides for the amplification of chicken antibody genes were designed by

Dr. Michael Hust according to published data[185], modified for the antibody gene phage display vector pHAL14 and kindly provided.

4.9 Enzymes

Enzyme/Buffer	Supplier
BSA 100 x	Fermentas
Calf intestine phosphatase (CIP)	Fermentas
Go Taq DNA polymerase	PROMEGA, Mannheim
Restriction endonucleases	Fermentas
Restriction-buffers NEB 2, 3, 4	Fermentas
T4 DNA Ligase	PROMEGA, Mannheim
T4 DNA ligase buffer 10x	PROMEGA, Mannheim
Trypsin	Sigma, München

4.10 Antibodies

Antibody	Application/Dilution	Supplier
goat-anti-mouseIgGFc-HRP 115-035-071	ELISA/Immunoblot 1:25,000	Dianova
mouse-anti-C-Myc-tag	ELISA/ 1:2000	AG Dübel
mouse-anti-pIII	Immunoblot/1:2000	MoBiTec, Berlin
goat-anti-humanIgGFc-HRP A 0170	ELISA Immunoblot	Sigma-Aldrich, München
goat-anti-mouseIgG Fc specif HRP A 2429	Immunoblot/1:10,000	Sigma-Aldrich, München
goat-anti-human IgG Fc AP 109-055-098		Dianova
goat-anti-chicken IgG HRP A9792		Sigma Aldrich, München

4.11 Software and databases

Software/Databases	Application	Reference
Vector NTI		
Multalin	amino acid alignment of antibodies	
NCBI Influenza virus resource	amino acid alignment of HA	
RCSB protein database	Protein structure modelling	
ITEM-Software	TEM/SEM	Olympus Soft Imaging Solutions, Münster, Germany
iMol version 0.40	Protein structure modelling	

4.12 Others

Product	Supplier
DNA ladders:	
Generuler™ 100bp DNA ladder	NEB
Generuler™ 1kb DNA ladder	NEB
Generuler™ 50bp DNA ladder, N3233	NEB
dNTPs	MBI Fermantas, St.Leon-Rot
Helperphage M13KO7	GE Healthcare, München
Protein ladders:	
Precision Plus Protein dual colo standard (250-10 kDa)	BioRad, München
Precision Plus Protein unstained standard (250-10 kDa)	BioRad, München
peqGoldIV	peqLab, Erlangen

5 Methods

5.1 General methods

Methods described herein have been applied several times within this thesis. All follow a general procedure described with given modifications.

5.1.1 Preparation of plasmid DNA

Plasmid DNA was extracted from growing *E.coli* over night cultures using DNA isolation kits from Macherey&Nagel according to manufacturer's protocols. For elution of DNA provided elution buffer (10 mM Tris, pH 8.0) or H_2O was used.

5.1.2 Precipitation of DNA

DNA was ethanol precipitated. Ligation samples were filled to a volume of 100 µL with H_2O. After addition of 10 µL 3 M sodium acetate buffer pH 5.2 and 275 µL 100% ethanol the sample was mixed well and incubated for 15 min at room temperature. Following a 5-15 min centrifugation at 4 °C and 16,000 xg the DNA-pellet was washed twice with 500 µL 70 % ethanol separated by a centrifugation step (5 min, 4 °C, 16,000 xg). The DNA pellet was air-dried and resuspended in an appropriate amount of H_2O .

5.1.3 Purifications of DNA from agarose gels or PCR reactions

Purifications of DNA from agarose gels or PCR reactions were performed using NucleoSpin® Gel and PCR Clean-up Kit (Macherey&Nagel) according to manufacturer's protocols.

5.1.4 Determination of DNA-concentration

Measurement of DNA-concentration was performed using NanoDrop spectrophotometer (Thermo Scientific) according to manufacturer's protocols.

5.1.5 Agarose gelectrophoresis

Gels with an agarose content of 0.8 – 2 % (w/v) and two different loading buffers (B-buffer and X-buffer) were used depending on expected DNA size. Size of DNA fragments was determined using a DNA-standard that was separated on the same gelcontaining ethidiumbromide. Separation was performed at 100 – 130 V and 300 mA. Detection in UV-light (λ = 312 nm). DNA was excised from agarose gels when indicated and purified using protocols Nucleospin Extract II kit (Macherey&Nagel).

5.1.6 DNA-amplification by PCR

PCR was used to amplify DNA for preparative and analytical purposes.

5.1.6.1 Colony PCR for analysis of scFv-antibody clones

Material from a single bacterial colony from agar plates served as template material. Annealing temperature depended on the oligonucleotides used and elongation time depended on the size of the amplified fragment (30 s either VH or VL antibody gene, 60 s complete scFv including VL and VH). Components and PCR-program for a typical colony PCR are shown in Tab 1

Table 1: Components and temperature program for a colony-PCR using pHAL14 vector DNA

Components	1 reaction [µL]	85 reactions [µL]
dH_2O	5.75	488.75
$MgCL_2$ (25 mM)	0.8	68
5x PCR buffer	2	170
dNTPs (10 mM each)	0.2	17
5' MH Lac Z-pro_f	0.6	51
3' MHgIII_r	0.6	51
GoTaq	0.048	4.08

Step	Temperature [°C]	Time [s]	
Denaturation	94	180	
Denaturation	94	30	30 cycles
Annealing	56	30	
Elongation	72	30 / 60	
Extention	72	600	
storage	16	∞	

5.1.6.2 PCR analysis of scFv-Fc antibody fusions

Three different vectors were used to obtain three different scFv-Fc antibodies. pCMV2.5-mIgG1Fc-XP, pCMX2.5-hIgG1Fc-XP and pCSE2.5-mIgG2cFc-Xp to obtain scFv-mIgG1Fc-, scFv-hIgG1Fc-, scFv-mIgG2cFc antibodies, respectively. Colony-PCR was performed after transformation of scFv-containing vectors into *E. coli* XL1-blue MRF' to identify clones containing the correct scFv-Fc antibody gene fragment with modifications given in Tab 2.

Table 2: Oligonucleotides used for the analysis of scFv-Fc antibodies after recloning into vectors pCMX2.5-hIgG2cFc-XP, pCMV2.5-mIgG1Fc-XP and pCSE2.5-mIgG2cFc-XP

Oligonucleotide	Annealing Temperature [°C]	Elongation time [s]
5' Tor-pCMV-mIgG01-FC-seq-f / 3' Tor-pCMV-mIgG01-Fc-seq-r	55	90

5.1.7 Restriction digest

Digests with one or two restriction enzymes per reaction were performed at the optimal temperature and buffersaccording to manufacturer's protocols. Analytical digestions were performed to analyse DNA cleavage products on an agarose or polyacrylamide gel; restriction products of preparative restrictions were used for cloning procedures.

The composition of a restriction digest depended on DNA-concentration and the enzyme used. A typical composition of a restriction digest is given in Tab 3.

Table 3: Typical composition of a restriction digest

Component	volume
DNA (e.g. 1 µg)	1 µL
Restriction enzyme (e.g. *Not*I) 10 U / µL	0.5 µL
10 x Reaction buffer (e.g buffer 3)	3 µL
dH_2O	addition to 30 µL
Total volume of restriction digest	30 µL

Further components like e.g. BSA were added and enzymes were heat inactivated after restriction digest where recommended.

5.1.8 Dephosphorylation of DNA

Restriction-digested plasmid DNA was dephosphorylated to prevent religation of the vector. Therefore, calf intestinal posphatase (CIP, Fermentas, 10 units/µL) was used. A total of 0.5 µL were incubated with restricted vector-DNA for 30 min at 37 °C, another 0.5 µL CIP were added and incubation time was repeated.

5.1.9 Ligation of DNA

T4-DNA-ligase (Promega) was used to ligate linear DNA fragments (inserts) and dephosphorylated vector DNA. Ligation was performed at 4-8 °C over night or 1-2 h at RT with e ftree-fold excess if insert over vector molecules. A typical ligation scheme is shown in Tab 4.

Table 4: Typical composition of a DNA-ligation

Component	Volume [µL]	amount
10 x ligation buffer	1	1
T4-DNA-ligase [1-3 u /µL]	0.5	1.5 U
Vector-DNA	1-2	50-100 ng
Insert -DNA	4-7	100-300 ng
dH_2O	addition to 10 µL	

5.1.10 Transformation of DNA into *E. coli*

Electroporation was used to transform electrocompetent *E. coli* XL1-blue MRF' (Agilent Technologies, Boeblingen, Germany) with plasmid-DNA or ligation samples. Up to 10 µL of purified plasmid-DNA or 1.5 µL of ligation sample was supplemented

with ice-cold H_2O and a maximum of 25 µL electrocompetent XL1-blue MRF' to a final volume of 50 µL. All mixing steps were perfomed on ice in precooled electroporation cuvettes. Electroporation was performed by an electric pulse of 1.7 kV for ~ 5 ms. 950 µL prewarmed (37 °C) SOC-medium was added immediately and the mixture was incubated for 1 h at 37 °C and 600 rpm. Aliquots or dilutions thereof were plated on agar-plates (dYT-agar supplemented with 100 mM glucose, 100 mg/L ampicillin and 20 mg/L tetracycline) and incubated over night at 37 °C to obtain single clone colonies of transformants.

5.1.11 Preparation and purification of phages after pannings

Single clone colonies grown on agar plates were carefully scraped off the plates in dYT-medium supplemented with glucose (100 mM) and ampicillin (100 mg/L). Optical density was measured at OD_{600} and an equivalent of OD_{600} 0.15 was used to inoculate 40 mLof the same medium composition. Bacterial culture was incubated at 37 °C, 250 rpm in shaking flasks until an OD_{600} of 0.5-0.6 was reached. 10 mL of the culture were used for superinfection with a 20fold excess of helperphage M13K07. Superinfection was performed for 30 min at 37 °C and 110 rpm in shaking flasks. Bacteria were pelleted by centrifugation at 3,220 xg and RT for 10min. Pellet was resuspended in 40 mL dYT-medium supplemented with ampicillin (100 mg/L) and bacteria were allowed to grow and produce phages over night at 30 °C and 250 rpm.

Over night cultures were filled in tubes and centrifuged at 10,000 xg and 4°C for 10 min. A total of 30 mL of the supernatant was transfered to a new tube and mixed with 7.5 mL 5 x PEG/NaCl by inverting and incubated for 30 min on ice. After an additional centrifugation step (10,000 xg and 4°C for 30 min) the phage containing pellet was resuspended in 0.8 - 1 mL phage dilution buffer and additionally centrifuged at 16,000 xg and 4°C for 10 min. Supernatant was filled into new reaction tubes and stored at 4-8 °C.

5.1.12 Determination of phage titres

A total of 100 µL of an exponentially growing *E. coli* XL1-blue MRF' culture at OD_{600} ~ 0.6 were added to a serial dilution of phage in a microtitre plate, plate was sealed with a breathable membrane and incubated for 30 min at 600 rpm in a microtitreplate shaker. 5 µL of each dilution were dotted twice and separately onto dYT-agar plates supplemented with glucose (100 mM) and ampicillin (100 mg/L). Plates were incubated over night at 37 °C and colony forming units were counted.

5.1.13 Production of soluble scFv-antibodies in a microtitre plate scale

To identify specific binders from clones resulting from the panning scFv-antibodies were produced in a 96 well microtitre plate (MTP) scale for the use in screening ELISA.

Single clone colonies from agar plates were used to inoculate 100 µL dYT-medium supplemented with glucose (100 mM) and ampicillin (100 mg/L) followed by an incubation for 6 h at 37 °C and 850 rpm in a MTP-shaker. 15 µL of this pre-culture were transfered to a new plate containing 185 µL dYT-medium supplemented with glucose (100 mM) and ampicillin (100 mg/L) and incubated for 1 -2 h at 37 °C and 850 rpm. Bacteria were pelleted by centrifugation at 3,220 xg, RT for 10 min, supernatant was discarded and 200 µL dYT-medium supplemented with ampicillin (100 mg/L) and 50 µM IPTG were added to allow the production of soluble scFv-antibodies during an over night incubation at 30 °C and 850 rpm.After over night production of soluble scFv antibodies plate was centrifuged at 3,220 xg, 4 °C for 10 min and supernatant was transfered into a new plate and used for screening ELISA.

5.1.14 Enzyme linked immunosorbent assay (ELISA)

To analyse the binding specificity of antibodies of different formats ELISA were performed. Target proteins were immobilized to ELISA plates in 50 mM sodium-carbonate buffer, pH 9.6, over night at 4 - 8 °C. After immobilization supernatant was discarded and wells were blocked with 2 % $MBPST_{0.05\ \%}$ or 2 % BSA $PBST_{0.05\ \%}$ (300 µL/well) for 1 h at RT. Wells were washed three times using $PBST_{0.05\ \%}$. All antibodies were incubated with immobilized target proteins in either 2 % $MBPST_{0.05\ \%}$ or 2 % BSA $PBST_{0.05\ \%}$ (100 µL/well) for a maximum of 1 h at RT. In screening ELISA 50 µL production supernatant of soluble scFv-antibodies were mixed with 2 % $MBPST_{0.05\ \%}$ and incubated on the target protein for 1 h at RT. Wells were washed three times with $PBST_{0.05\ \%}$ after each incubation step. Final incubation step was always performed using an HRP-labeled secondary antibody. For staining a mixture of TMB-A and TMB-B (20:1) was used (100 µL/well) and incubated for 10 min except as noted otherwise. Staining reaction was stopped with 1 M H_2SO_4 (100 µL/well). Measurement of optical density was performed at wavelength λ = 450 nm with reference at OD_{600} using an MTP reader.

5.1.15 SDS-PAGE

Proteins were separated using a 12 % SDS-containing polyacrylamide gel (Tab 5.). 5 x Laemmli-buffer (including β-mercaptoethanol for reducing conditions, without β-mercaptoethanol for non-reducing conditions) was added to protein samples that were heated for 8 min at 94 °C (reducing) or 56 °C (non-reducing). Proteins were separated at 25 mA, 300 V for 45 – 50 min.

Table 5: Composition of a 12 % separation and a 4 % stacking gel for SDS-polyacrylamide gel

Component	Volume [µL]	
	Separating gel 12 %	Stacking gel 4 %
dH_2O	1300	1000
30 % Bisacrylamide	1600	260
1.5 M Tris HCl, pH 8.8	1000	-
1 M Tris HCl, pH 6.8	-	200
10 % SDS	40	15
10 % APS	40	15
TEMED	2	2

5.1.16 Coomassie staining of protein gels

After SDS-PAGE protein gels were transfered into a beaker, covered with Coomassie-staining solution, boiled using a microwave and incubated for 5 min. Staining solution was removed followed by 2 -3 washing steps using H_2O and heating using the microwave. Stained gels were stored in H_2O.

5.1.17 Silver staining of protein gels

Silver staining of protein gels were performed using silver stainig kit (GE Healthcare) according to manufacturer's procedure.

5.1.18 Immunoblots

Proteins separated on an SDS-gel were blotted onto a PVDF-membrane using a semidry-blotting procedure. PVDF-membrane was incubated for 2 -3 min in methanol. Blotting of proteins onto the membrane was performed at 0.45 mA/cm^2 gel, 300 W, 20 V for 25 min. After blotting the membrane was blocked for 10 min in 2 %

Tween PBS. The blocked membrane was washed three times in PBS, 5 min each. Target protein specific antibodies were incubated in $MPBST_{0.05}$ with the membrane for 1 h at RT. Secondary antibody was either HRP- or AP labeled and incubated in $MPBST_{0.05}$ for 1 h at RT. Each incubation step was followed by three washing steps using $PBST_{0.05\ \%}$, 5 mineach. Staining of AP-labeled secondary antibodies was performed using AP-substrate buffer and NBT/BCIP (each 1:100 in substrate buffer). For staining of HRP-labeled secondary antibodies DAB with its respective substrate buffer and 37 % H_2O_2 (1:10,000). Staining reactions were stopped with H_2O. Commercial antibodies used for immunoblot and their appropriate dilution for this application are summarized in section 3.10.

5.1.19 Hemagglutination inhibition test (HI)

Using a hemagglutination test **(HA test)** viruses can be detected that are able to agglutinate erythrocytes via virus surface proteins. In this assay virus amount is defined as hemagglutination units that are calculated in log 2.

In a hemagglutination inhibition test **(HI)** antibodies can be analysed with regard to their ability to inhibit hemagglutination due to binding to hemagglutinin and thereby masking sites necessary for the agglutination of erythrocytes. HI-positive antibodies have a high potential to serve as neutralizing antibodies.

Chicken blood was supplemented with sodium citrate or heparin to prevent agglutination. Erythrocytes were washed in PBS three times and centrifuged at 1500 rpm for 10 min at 4 °C after each washing step. From the sediment a 1 % (v/v) chicken erythrocyte solution was prepared und used for this assay. For positive control the following antigen was used: Newcastle Disease Virus (NDV) Ulster (4^{th} egg passage, 11.9.2007, inactivated antigen, K-number 3709; FLI) that gives a hemagglutination titre of 8 (+/-1 log_2 step). The assay was performed in round-bottom MTP plates.

For the HI test first of all the virus amount needs to be fixed to 4 hemagglutination units (HAU 4, dilution 1:16) for all viruses to be assayed. A total amount of 25 µL NaCl 0.85 % (w/v) per well was mixed with an equal amount of virus containing solution and eleven similar serial dilutions were performed (dilutions 1:2 to 1:4096). A total of 25 µL of a 1 % chicken erythrocytes solution is added per well and incubated for 30 min at RT. The last virus dilution with positive hemagglutination (slow sedimentation compared to erythrocyte control, formation of a “cobweb”-like

structure) is used to calculate HAU 4. Accuracy of HAU 4 determination was controlled by a control HA test. Each HA test required a positive control (NDV Ulster titration) and a negative control (erythrocytes without virus).

Serial antibody dilutions of 1:2 with virus dilution at HAU 4 were incubated for 1 h at RT. After the additon of 25 µL of a 1 % chicken erythrocyte solution and an additional incubation for 30 min at RT the hemagglutination inhibition concentration of the assayed antibody can be calculated from the last well were agglutination is inhibited (no hemagglutination detectable).Each HI test required a positive control (virus with antibody) and two negative controls (erythrocytes without virus and antibody as well as erythrocytes with antibody dilutions).

5.1.20 Immunoperoxidase assay (IPMA)

Two different cell lines were used for this cell based ELISA. Cell lines and viruses as were provided by the FLI.

A swine testis cell line (STOMA24) was used for the infection with low pathogenic influenza viruses presenting viral proteins – among them hemagglutinin on their surface upon infection. To present hemagglutinins of highly pathogenic influenza viruses on the cell surface recombinant insect cells (High Five) constantly expressing the respective hemagglutinin were used.

The amount of virus to be used had to be titrated first to guarantee an infection of 5 - 15 % of the cells per well. From a confluent cell culture of adherent swine testis cells in a middle-size cell culture flask medium was removed, wells were washed using 2 – 3 mL trypsin containing solution (“ATV-D”, FLI) and detached using 5 mL ATV-D and an incubation period of 5 min at 37 °C. Serum free medium (“5ZB-D”, FLI) supplemented with G418 EK [400µg/mL], Hygromycin EK [200µg/mL] and Doxycycline [300ng/mL] was used to give a total volume of 40 mL. A total volume of 100 µL cell suspension was added to 100 µL of the respective virus solution per well and virus replication was allowed for 2 days at 37 °C and 5 % CO_2.

After infection medium was removed and cells were washed using 100 µL PBS per well. Fixation of the cells was performed using 100 µL fixation solution (40 % methanol, 60 % acetone) per well during an incubation of 5 min. Fixation solution was removed and cells were carefully air-dried. Fixed cells were incubated for 30 min at RT with 50 µL TBST per well supplemented with 0.03 % (v/v) H_2O_2. After removing the supernatant cells were washed in TBST followed by the incubation of the first

antibody (500 ng scFv-hIgG1Fc antibody in 50 µL TBSTper well; 1 h, RT). After three washing steps (each 250 µL TBST) wells were incubated with a secondary HRP-conjugated goat-anti-humanFc antibody (1:500 dilution in TBST) for 1 h at RT. After three washing steps (each 50 µL TBST) wells were incubated for 15-30 min at RT in 50 µL staining solution (0.05 M sodium acetate pH 5.0 containing 0.03 % (v/v) H_2O_2 and 5 % (v/v) ACE-stock solution) per well. Staining solution was removed and wells were washed using 50 µL H_2O per well. Per antibody and virus four independent wells were analysed.

5.1.21 Virus Neutralization assay (VN)

$MDCK^+$ cells were used for virus neutralization and assays were performed in 96 well MTP plates in serum free medium ("5ZB-D", FLI). Cells, medium and all supplements were provided by the FLI. $MDCK^+$ cells were prepared to be seeded in 96 well MTP plates according to the following procedure:

From a 150 cm^2 flaskof confluent $MDCK^+$ cells medium was removed and cells were washed in 5 mL ATV-D (trypsin containing solution). Supernatant was removed and cells were incubated in 5 mL ATV-D at 37 °C, 5 % CO_2 for no longer than 50 min until cells were detached. Cell suspension was transfered to a 15 mL tube supplemented with 2 mL ("ZB 5b 5% FCS", FLI) and centrifuged at 800 rpm and RT for 2 min. Supernatant was removed, $5x10^4$ – $1x10^5$ cells per well were seeded in serum containing medium and incubated for one day at 37 °C, 5 % CO_2.

For all viruses to be used 100 $TCID_{50}$ (100 fold the amount of virus needed to infect 50 % of all cells) had to be determined since this virus amount was used for virus neutralization assays. Virus amount had to be confirmed via backtitration at the day when VN assay was performed.

Log 2 serial antibody dilutions were performed in serum free medium ("5ZB-D", FLI) and 50 µL of each dilution step was preincubated with 50 µL virus suspension at 100$TCID_{50}$ for 1 h at RT. Cells in 96 well plates were washed with 100 µL serum free medium ("5ZB-D", FLI). 50 µL of serum free medium supplemented with trypsin (TPCK, FLI) at a final concentration of 2 µg/mL was added together with the 100 µL preincubated antibody/virus mixture. Plates were incubated for 72 h at 37 °C and 5 % CO_2 before wells were analysed using a microscope. Four separate wells were analysed per antibody concentration and virus. Antibody was regarded to be neutralizing when no cytopathic effect could be observed in all four wells.

Due to unexpected negative VN-results for antibody JM7_8 and to reduce potential negative effects of trypsin on antibody stability described standard procedure was altered according to the following protocol:

After cells were washed antibody/virus mixture was added to the cells and infection of the cells was allowed for 6 h at 37 °C and 5 % CO_2. Only then trypsin containing medium was added and plates were incubated for 66 h at 37 °C and 5 % CO_2.

5.2 Immunization of chickens

Specific Pathogen-Free (SPF) chickens were immunized at the age of three weeks with a vaccine based on the virus isolate A/duck/Anhui/2006 (Chinese commercial inactivated vaccine Re-5). Three weeks later these birds were infected with A/chicken/Egypt/0879 - NLQP/2008 H5N1 virus (10^6 $TCID_{50}$/bird). All immunized birds survived without developing clinical symptoms until day 14 after second immunization[120]. The birds were then sacrificed and heparinised blood samples and spleens were obtained for isolation of lymphocytes. All experimental procedures followed official German animal welfare regulations (permission LALLF 127M-V/TSD/7221.3-2.1-031/09) and were perfomed byDr. Christian Grund at the FLI.

5.3 Purification of influenza viruses

Virus A/teal/Föhr/Wv632/05 (H5N1, LP) was propagated in 9 to 11 days old SPF embryonated chicken eggs and inactivated using beta-propriolactone according to an OIE protocol. Allantoic fluid containing the virus was harvested after decapping the egg and centrifuged at 3500 rpm for 30 min. Clear supernatant was loaded onto a sucrose gradient (60%, 35% (w/v) with 5mL of each sucrose fraction; sucrose dissolved in Tris-buffer (100 mM Tris pH7.4, 3mM EDTA pH7.2) and centrifuged for 2 hours at 27,000 rpm and 4°C using a Beckmann LE-70 ultracentrifuge (rotor SW32) with ultraclear centrifuge tubes (38.5 mL, SW28/32). Virus containing band was diluted 1:2 in Tris buffer and loaded onto a second sucrose gradient (60/50/40/30% (w/v) sucrose; 7 mL each fraction) and centrifuged over night at 27,000 rpm, 4°C. White, virus containing band was diluted 1:5 in 0.85% (w/v) NaCl and pelleted at 27,000 rpm, 4°C for 90 min. Virus pellet was finally resuspended in 0.85% NaCl.

5.4 Generation of chicken immune libraries

Two different anti-H5 chicken immune antibody-libraries were constructed i) from spleen (referred to as KFC_AIV2_S) and ii) from peripheral blood lymphocytes

(referred to as KFC_AIV2_B).

5.4.1 Isolation of RNA

Isolation of RNA was performed by the FLI using Qiagen RNeasy RNA-isolation kit according to FLI standard protocols.

Isolated RNA was shipped at 4 – 8 °C and further used at the day of delivery.

5.4.2 Reverse transcription

RNA was reverse transcribed using Superscript-II (Invitrogen, Darmstadt, Germany) and random hexamer oligonucleotides (denaturation 70°C for 5 min, synthesis 10 min 25°C, 50 min 42°C and finally 15 min 70°C).

5.4.3 cDNA-synthesis

The RNA content was measured and RNA amount used for cDNA synthesis was calculated (values not shown). Mix I (consisting of random oligonucleotides and dNTP-mix) was added to RNA, mix was incubated at 70 °C for 5 min to denature RNA followed by a 5 min incubation step on ice. Mix II (consisting of 5xFS-buffer and DTT) was added afterwards and 1 µL Superskript II Reverse Transkriptase (200 U/µL)per sample was added separately. A three step synthesis program was performed (10 min at 25 °C, 50 min at 42 °C, 15 min at 70 °C). Storage of cDNA was avoided and immediately processed for amplification of chicken antibody genes via PCR.

Table 6: Composition and procedure for cDNA synthesis from total RNA isolated from chicken blood (B) and spleen (S) to construct anti-H5 chicken immune antibody libraries KFC_AIV2_S and KFC_AIV2_B

Components	KFC_AIV2_S	KFC_AIV2_B
sample number	10-13	1-9
total RNA	9 µL	18 µL
add Mix I,		
Components Mix I	25 µL each	38 µL each
random Oligonucleotides (dN6) 10 µM	11.25 µL	23.75 µL
dNTP-Mix (2.5 mM each)	22.5 µL	47.5 µL
7.5 µL per PCR-tube; 5 min at 70 °C; 5 min on ice		
add Mix II,		
Components Mix II	25 µL each	38 µL each
5xFS-buffer	22.5 µL	72.2 µL
0,1 M DTT	11.25 µL	36.1 µL
	7.5 µL each	**11.4 µL each**
Superskript II Reverse Transkriptase (200 U / µL)	1 µL	1 µL
10 min at 25 °C, 50 min at 42 °C, 15 min at 70 °C		

5.4.4 Amplification of chicken antibody genes – first PCR

Chicken specific IgG-oligonucleotides were used to amplify variable (V) heavy (H) and light (L) chains in a first PCR using oligonucleotides shown in section 4.8. A total of 26 PCR samples for 9 cDNA_samples from peripheral blood lymphocytes (9 individual chickens) and 4 cDNA-samples from spleen-RNA (4 individual chickens) were performed.

Table 7: Pipetting scheme for PCR to amplify antibody gene fragments from cDNA to construct chicken immune antibody libraries

	28 reactions
dH_2O	912.8 µL
$MgCl_2$ (25 mM)	112µL
5x Go-Taq-Puffer	280 µL
dNTPs (10 mM each)	28 µL
GoTaq 5U/µL	11,2 µL

A total of 28µL of the respective oligonucleotide for the amplification of chicken VH and VL antibody chains (10 µM) was added to the PCR mixture. Both VL- and VH-PCR-mixes were aliquoted in PCR tubes. 48.6 µL per tube and 1.6µL of the respective cDNA was added to each tube. Thus two samples (VH and VL) were obtained for each amplified cDNA. The following PCR-program was run: 3 min 94°C, 30 s 94°C / 30 s 60°C / 30 s 70°C / 10 min 72°C. A total of 25 cycles was performed.

PCR samples were separated on a 1.5 % agarose gel via gel-electrophoresis. Gel was run for 40 min at 100V. DNA bands of correct size (VL 350bp, VH 390bp) were excised and purifiedusing MNNucleospin Kit for each PCR sample separately according to manufacturer's procedure. Final volume for elution was 35 µL for each sample.

5.4.5 Introduction of restiction sites – second PCR

Restriction cloning sites (*Nco*I/*Hind*III for VH and *Mlu*I/*Not*I for VL) were inserted by a second PCR. Used oligonucleotides are listed in section 4.8.

A total of 1µL of the appropriate 10 µM oligonucleotide stock (VH: MHChickVH_f and MHChickVH_r2; VL: MHChickVL_MLuI_f and MHChickVL_NotI_r) was used per reaction. A total of 30 ng PCR-product from first PCR was used for samles obtained from blood and 50 ng for those obtained from spleen. Final volume of each PCR-reaction was 50 µL.

A total of 25 PCR cycles were run in a two step-PCR. First 10 cycles were run with lower annealing temperature - 3 min 94 °C, 30 s 94 °C / 30 s 60 °C for VH and 55 °C for VL, 30 s 70°C; the following 15 cycles were run at higher annealing temperature: 3 min 94 °C, 30 s 94 °C / 30 s 67 °C for VH and and VL, 30 s 70 °C followed by a 10 min 72 °C step.

PCR samples were separated on a 1.5 % agarose gel via gel-electrophoresis for 40 min at 100V. DNA bands of correct size (VL 350bp, VH 390bp) were excised and purifiedusing MNNucleospin Kit for each PCR sample separately according to manufacturer's procedure. Final volume for elution was 37 µL for each sample.

5.4.6 Restriction digest and ligation of antibody gene fragments and pHAL 14 vector

Amplified chicken antibody gene fragments were digested using appropriate restriction enzymes (VL – *Hind*III/*Nco*I and VH – *Mlu*I/*Not*I, according to Tab 8). A total of 5 µg pHAL14 vector and 1 µg insert VL and VH were digested. VH and VL samples of blood and spleen origin were pooled to give pools of VH- and VL – PCR fragments of blood and splenic origin each. In this work VH - antibody gene fragments were cloned first into pHAL 14 phage display vector. Restriction digests were performed as shown in Tab 8 and fragments were cloned into pHAL 14 vector according to ligation shown in Tab 9 using 1 µg vector DNA and 250 ng insert DNA over night at 4-8 °C and inactivated for 10 min at 65 °C. Following inactivation DNA was precipitated and transformed.

Table 8: Restriction digest of VH antibody gene fragments from blood (B) or splenic (S) origin and pHAL 14 antibody phage vector

Components	pHAL14 5µg [µL]	VH B 1.5µg [µL]	VH S 1.59µg µL]
DNA	5	47.25	60
10 x NEB buffer 2	10	10	10
*Nco*I	2.5	2	2
dH_2O	82.5	40.75	28
incubation for 3 h at 37 °C heat inactivation 10 min at 65 °C			
10 x NEB buffer 2	0.5	0.5	0.5
*Hind*III (20 U / µL)	5	5	5
incubation over night at 37 °C			

Table 9: Ligation of VH antibody gene fragments from blood (B) or splenic (S) origin into pHAL 14 phage display vector

components	VH B [µL]	VH S [µL]
vector pHAL 14	10	10
insert	3.7	5.1
10x ligase buffer	10	10
T4 DNA ligase	1	1
H_2O	75.3	73.9

Table 10: Restriction digest scheme of VH-pHAL14 and VL antibody gene fragments prior cloning VL fragments into VH-pHAL14 phage display vector

components	VHpHAL14 B 5µg [µL]	VHpHAL14 S 5µg [µL]	VL B 2µg [µL]	VL S 2µg [µL]
vector	3.5	3.6	-	-
PCR	-	-	55.8	48
H_2O	80.5	80.4	30.8	37
10 x NEB3 buffer	10	10	10	10
*Mlu*I [10 U / µL]	2.5	2.5	1	1
incubation 1 h at 37 °C				
100 x BSA	1	1	1	1
*Not*I [10 U / µL]	2.5	2.5	1	1
incubation over night at 37 °C				

Ligation of VL antibody fragments and pHAL 14 vector containg VH antibody gene fragments was performed as shown in Tab with 250 ng insert (VL) and 1 µg vector DNA.Following inactivation of the ligation (10 min, 65 °C) DNA was precipitated and transformed via electroporation in *E. coli* XL1-*Blue* MRF' (Agilent) with three shots each 10 µL DNA, pHAL 14 vector DNA including complete scFv chicken antibody gene fragments was produced in *E. coli* XL1-*Blue* MRF' and purified.

5.4.7 Packaging of chicken antibody genes into phage libraries

To obtain phage libraries in which chicken scFv-antibodies are fused to the pIII-phage coat protein and thus presented on the surface of the phage single clone colonies were scraped off agar plates using dYT-medium supplemented with 100 mM glucose and ampicillin (100 mg/L). Optical density was measured at OD_{600} and an equivalent of OD 0.15 was used to inoculate two cultures of each 400 mLof the same medium composition. Bacterial culture was incubated at 37 °C, 250 rpm in 2 L shaking flasks until OD_{600} of 0.5 was reached. Two cultures of 40 mL were used for superinfection with a 20fold excess of helperphage M13K07 supernatant. Superinfection was allowed for 30 min at 37 °C and 110 rpm in 500 mL shaking flasks. Culture was filled in 50 mL tubes and bacteria were pelleted by centrifugation at 3,220 xg and RT for 10min. Pellet was resuspended in 400 mL dYT-medium supplemented with ampicillin (100 mg/L) and bacteria were allowed to grow and

produce phages over night at 30 °C and 250 rpm in 2 L shaking flasks. Remaining culture volume from 400 mL pre-culture was aliquoted each 45 mL in 50 mL tubes and centrifuged at 3,220 xg for 10 min at RT. Supernatant was discarded and pellet was resuspended in 500 µL dYT-medium supplemented with ampicillin (100 mg/L) and 250 µL 80% glycerol(v/v). The mixture was frozen in twist-off caps in liquid nitrogen to be stored at - 80 °C.

5.4.8 Purification of chicken immune library antibody phages

Purification of chicken immune library antibody phages was performed with modifications. After centrifugation of over night cultures, 200 mL of the supernatant were transfered into a new SLA 3000 tube and mixed with 50 mL 5 x PEG/NaCl by inverting and incubated for 30min on ice. After an additional centrifugation step (10,000 xg and 4°C for 30 min) pellet was resuspended in 10 mL phage dilution buffer, and additionally centrifuged at 20,000 xg and 4°C for 10 min. To the supernatant 5 mL 5 x PEG/NaCl were added, mixed and incubated for 1 h on ice. Supernatant was cleared by an additional centrifugation step (30 min, 4 °C, 20,000 xg) and each pellet was resuspended in 1mL phage dilution buffer. After the last centrifugation step (16,000 xg, 4°C, 10 min) supernatant was stored at 4 – 8 °C.

5.5 Immobilization of H5

For the immobilization of the two different recombinant H5 antigens four different surfaces were used resulting in five different immobilization procedures. A short overview on H5 immobilization procedures is given in Fig 14.

5.5.1 Immobilization of human H5 to carboxylic beads

Recombinant human H5 A/Anhui/1/2005 protein was immobilized to Dynabeads® M270 carboxylic beads according to manufacturer's procedures. A total of 10 µL beads was activated using a mixture of 100 µL 0.1 M N-hydroxysuccinimide (NHS) and 100µL 0.5 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) bya 8 min incubation at RT. After two short wash steps with 10 mM sodium acetate buffer, pH 5.0, 500 ng of recombinant H5 were immobilized in 10 mM sodium acetate buffer, pH 5.0 for 20 min at RT in a final volume of 120 µL. Subsequently, beads were washed with PBS and blocked with 50mM ethanolamine for 1h at RT. Finally beads were washed with PBST (PBS containing 0.05% (v/v) Tween-20) and blocked with 1 % BSA PBST for 1h at RT.

5.5.2 Immobilization of human H5 to epoxylic beads

Recombinant human H5 A/Anhui/1/2005 protein was immobilized epoxylic magnetic beads using two different coupling buffers: PBS, pH 7.4 or 100 mM sodium borate, pH 8.6. Immobilization was performed at 6 °C in a thermomixer, shaking at 800 rpm using a 2 mL round bottom reaction tube. Following incubation beads were washed twice with the respective coupling buffer and bead surface was saturated using 50 mM ethanolamine in PBS (1 mL per reaction tube) for 1 h at RT, rotation. Following saturation beads were washed twice using PBST and blocked with blocking buffer (2 % MPBST or 2 % BSA PBST) for 1 h at RT, rotating.

5.5.3 Immobilization of human H5 to ELISA plates

Recombinant human H5 A/Anhui/1/2005 protein was immobilized to ELISA plates in 50 mM sodium carbonate buffer pH 9.6 over night at 4-8 °C. For ELISA using scFv-Fc antibodies 50-75 ng/ well were immobilized. Following immobilization wells were blocked with blocking buffer (2 % MPBST or 2 % BSA PBST) for 1 h at RT, 300 µL/well.

5.5.4 Immobilization of avian H5 to streptavidin beads

Biotinylated recombinant trimeric avian H5A/Canada goose/Germany/71/06protein was immobilized to Dynabeads® M280 Streptavidin (Invitrogen, Darmstadt, Germany) according to manufacturer's procedures. Briefly, 10 µL streptavidin beads were incubated with in 200 µL baculovirus-expression supernatant for 1h at RT and then blocked with PBST supplemented with 1% (w/v) skim milk for 1h at RT.

5.6 Pannings

In general:

A total of two to three successive rounds of panning were performed. First panning round: A total of $5x10^{11}$ cfu (colony forming units) of each antibody phage library was incubated on immobilized H5 target for 60 - 80 min at RT in 1% BSA PBST. Beads were washed 15 times with 800 µL PBST. Bound phages were eluted from beads by incubation with 200 µL 10 µg/mL trypsin for 30 min at 37°C shaking at 600 rpm. A total of 5 mL *E. coli*XL1-blue MRF' were inoculated in dYT medium supplemented with 20 µg/mL tetracycline at 250 rpm at 37°C were infected at an OD_{600}= 0.5 – 0.6 with the eluted phage by incubation for 30 min at 37°C and shaking at 110 rpm. Cells were plated onto dYT-agar plates supplemented with 100 mM glucose, 100 µg/mL ampicillin and 20 µg/mL tetracycline (dYT-GAT). Plates were incubated over night at

37°C. For the following two panning rounds 1 % MPBST and 1% BSA PBST was used for blocking, respectively and 25 or 20 washings steps were performed respectively.

5.6.1 Pannings using human naïve antibody libraries

5.6.1.1 Pannings on human H5 target immobilized to ELISA plates

Human recombinant H5 A/Anhui/1/2005 target was immobilized as described in section 4.5.3. Pannings were performed using naïve human antibody libraries HAL7 and HAL4 (hyperphage packaged).

First round of panning: A total of 250 ng H5 protein was immobilized per well and 4 wells were used. A total of $5x10^{11}$cfu phages were used per library, phage were pooled and first preincubated for 15 min in 1 % BSA PBST. Second preincubation step was performed in 1 % BSA PBST blocked ELISA plate wells (300 µL 2 % BSA PBST per well for immobilization 1 h, RT) using 100 µL preincubated phage per well. After incubation of phage on the target for 90 min at RT shaking, wells were washed 10 x PBST (bottom-wash program using Columbus ELISA washer) and elution of phage were performed using 100 µL 10 µg/mL trypsine per well for 30 min at 37 °C and 800 rpm in a MTP shaker. Second round of panning: A total of 500 ng H5 protein was immobilized per well and 2 wells were used. A total of $7.6x10^{11}$cfu phages from first panning round with 1 % MBST used as blocking buffer. After incubation of phage on the target for 60 min at RT shaking wells were washed 30 x PBST. Third round of panning: was performed similar to the second round with $4x10^{9}$ cfu phages from second panning round and 1 % BSA PBST used as blocking buffer. After incubation of phage on the target for 90 min at RT shaking wells were washed 20 x PBST

5.6.1.2 Pannings on human H5 target immobilized to magnetic beads

Human recombinant H5 A/Anhui/1/2005 target was immobilized as described in section 4.5.1 and 4.5.2. Pannings were performed using naïve human antibody libraries HAL7 and HAL4 (hyperphage packaged).

First round of panning: A total of 500 ng H5 protein was immobilized to carboxylic and epoxylic magnetic beads, 10 µL each. A total of $5x10^{11}$cfu phages were used per library, phage were pooled and first preincubated in 800 µL 1 % BSA PBST for 15 min, at RT, rotating. Blocked target beads were incubated with preincubated phage for 1 h at RT, rotating. Beads were washed 15 times with each 800 µL PBST within 30 min. Elution and phage recovery was performed as described under 5.6 – in

general. Second round of panning: A total of 250 ng H5 protein was used and $8x10^{10}$ cfu phages from first panning with 1 % MPBST as blocking buffer. Beads were washed 20 times. Third round of panning: was performed similar to procedure for the second panning round. Incubation and blocking buffer was 1% BSA PBST and phage input was ~ 5 x 10^9 cfu.

5.6.2 Pannings using chicken immune libraries

Two different H5 antigens were used for these series of pannings. Recombinant human H5 A/Anhui/1/2005 was immobilized to carboxylic beads and recombinant avian H5 antigen A/Canada goose/Germany/71/06 to streptavidin beads. A summary of the panning procedure is given in Fig 12.

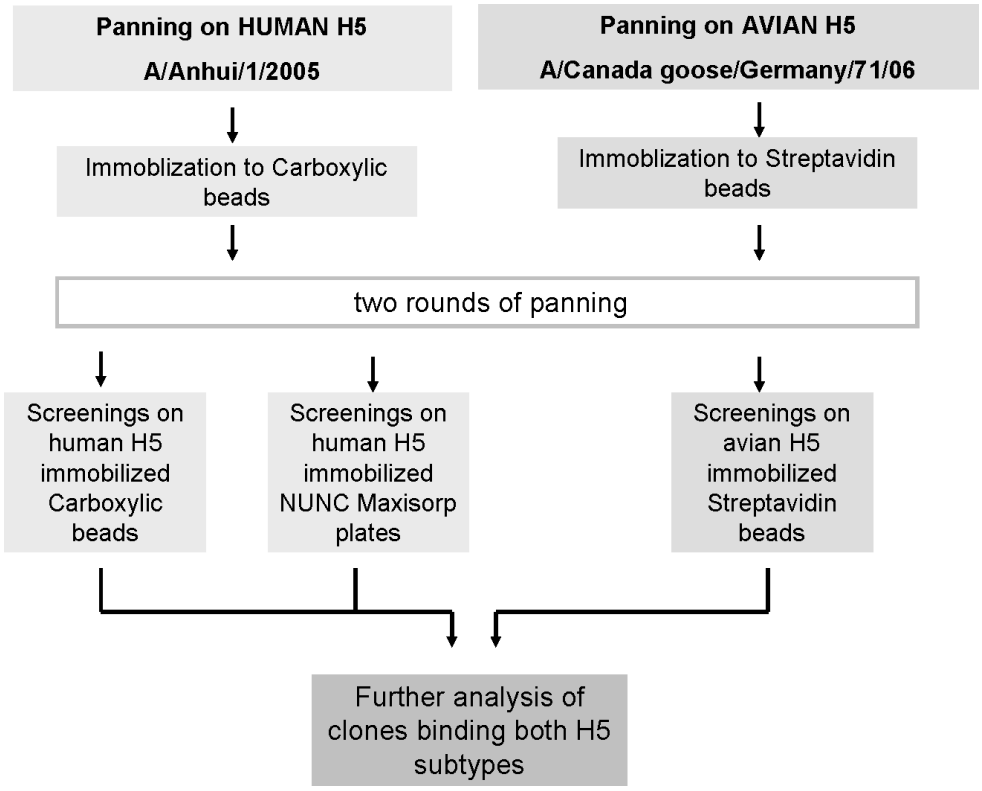

Figure 12: Scheme of panning campaigns to identify anti-H5 chicken antibodies

5.6.2.1 Panning on recombinant human H5

A total of two successive rounds of panning was performed. First panning round: A total of $5x10^{11}$ cfu of each phage immune library KFC2_AIV_S and KFC2_AIV_B was incubated separately on H5 beads for 80 min at RT in 1% BSA PBST. Afterwards, beads were washed 15 times with 800 µL PBST. Second panning round:Amplified phage preparations from first panning round (KFC-AIV2-S: $1x10^{11}$ cfu, KFC_AIV2_B: $5x10^{10}$ cfu) were incubated with H5 antigen coated beads in 1 % MPBST. Beads were washed 25 times. After two panning rounds single clones were isolated.

5.6.2.2 Panning on recombinant avian H5

Panning procedure was similar to procedure described in 4.6.2.1 with modifications.

Streptavidin beads were coated with 200 µL biotinylated avian trimeric H5 A/Canada goose/Germany/71/05 from cell culture supernatant for 60 min at RT. Beads were washed with PBST and blocked in 2 % MPBST in the first panning round or in 2 % BSA PBS in the second round of panning. The immune libraries were diluted in blocking buffer and incubated with blocked andH5 antigen coated beads, 1 h at RT, rotating for each incubation step. A total of 15 or 25 washing steps was performed in the first or second panning round, respectively.

5.7 Screening for scFv antibodies via ELISA

In general: For screening ELISA scFv antibodies were produced in microtitre plates. H5 target antigens were immobilized to various surfaces as described in section 4.5. A total of 50 µL of scFv antibody production supernatant was incubated on immobilized target in blocking buffer (either 1 % MPBST or 1 % BSA PBST) to a final amount of 100 µL per well for 1 h at RT. For bead immobilized target 1 µL beads was used per well. Following this washing step wells were incubated with 100 µL mouse-anti-c-myc antibody in a final concentration of 1:1000 – 1:2000 depending on the antibody preparation (in house production) using blocking buffer for 1 h at RT. An HRP-conjugated goat-anti-mouse Fc antibody 1:1000 diluted in blocking buffer was used for secondary antibody. Each antibody incubation was followed by a washing step in PBST (three times) using either Tecan Columbus pro washer (for MTP immobilized target) or Tecan hydroflex bead washer (for bead immobilized target). Staining was performed using TMB (TMB A/TMB B 20:1, 100 µL per well) for 10 min, reaction was stopped by addition of 100 µL 1 N H_2SO_4. $OD_{450\ nm}$ measurements were performed at (reference at 600 nm) using a 96 well-plate reader.

5.8 Cloning of scFv-antibody genes into scFv-Fc-antibody format

Three different scFv-Fc expression vectors were used – pCMX2.5-hIgG1Fc-XP, pCMV2.5-mIgG1Fc-XP and pCSE2.5-mIgG2cFc-XP to express different scFv-Fc antibodies scFv-humanIgG1Fc, scFv-mouseIgG1Fc and scFv-mouseIgG2cFc, respectively.

ScFv-antibody fragment was isolated from pHAL14 vector using restriction enzymes *Nco*I and *Not*I. Restriction digest was loaded onto a 1 % agarose gel, scFv-DNA

insert was separated from vector background by gel electrophoresis and scFv-DNA insert, ~ 900 bp in size, was excised from gel and purified using Macherey&Nagel Nucleospin Gel and PCR clean up kit (MN DNA purification kit). ScFv-Fc expression vectors were treated with restriction enzymes *Nco*I and *Not*I, purified by MN DNA purification kit using a modified procedure according to manufacturer's recommendation and dephosphorylated. ScFv-DNA insert and dephosphorylated vector were ligated (~ 40 ng of vector DNA and ~ 40 ng of insert DNA), inactivated and transformed into *E. coli* XL1-blue MRF'. Following an incubation over night at 37 °C single cones were used to perform colony PCR to identify clones having incorporated the respective scFv-Fc expression vector including an scFv-fragment. Bacteria containing the insert of correct size were used to amplify plasmid DNA and plasmid DNA was purified using MN Nucleobond Xtra Midi Kit according manufacturer's protocols. Precipitated DNA was eluted in H_2O and used for transfection of HEK cells.

5.9 Production of scFv-Fc antibodies

ScFv-Fc antibodies were produced in mammalian cells – either adherent HEK293T or in HEK293-6E in shaking.

5.9.1 Expression of scFv-Fc antibodies in HEK293T cells

Confluent HEK293T plates were split 1:4 using DMEM medium supplemented with 8 % (v/v) FCS and 1 % penicillin/streptomycin 13 mL per plate. 10 µg DNA was mixed with 400 µL DMEM medium in one well of a 12 well plate and 400 µL DMEM was mixed with 80 µL PEI [1 mg/ mL]] in a second well. Content of both wells were mixed and incubated for 20 -30 min at RT. Transformation mixture added to the cells and plates were incubated at 37 °C and 7 % CO_2. After 24 h medium was removed and DMEM supplemented with 4 % (v/v) low IgG FCS and 1 % penicillin/streptomycin was added. Culture supernatant was harvested every day and fresh medium was added. Production of scFv-Fc antibodies was continued for up to 10 days, harvested culture supernatant was stored at 4 - 8 °C.

5.9.2 Expression of scFv-Fc antibodies in HEK293-6E cells

ScFv-Fc antibodies were produced in shaking culture using HEK293-6E cells. Optimal cell number is 1.7 -2 million cells per mL, culture medium is FreeStyle F17 expression medium (GIBCO) supplemented with 7.5 mM glutamine and 0.1 % (w/v) pluronic F68. Transfection procedure is described for a 100 mL standard production

scale. 100 µg DNA was mixed with 3 mL medium in one well and 3 mL medium was mixed with 250 µL PEI [1 mg/ mL] in a second well. Content of both wells were mixed by slowly pipetting up and down and incubated for 20 -30 min at RT. Transfection mixture was added to shaking cell culture and cells were incubated at 37 °C, 5% CO_2 and 111 rpm. Two days after transfection 50 mL medium F17 complete containing 0,5% (w/v) TN1-tryptonewas added. Production of scFv-Fc antibodies was allowed for up to six days.

5.10 Purification of scFv-Fc antibodies

ScFv-Fc antibodies were purified using Profinia™ (BIO-RAD) protein A affinity gel chromatography columns. Cell culture supernatants were centrifuged at 2000 rpm for 10 min. Prolonged storage of cell culture supernatants was avoided but if necessary 0.5 % (v/v) low IgG FCS wasadded to reduce degradation of scFv-Fc antibodies by proteases. Cell free supernatants were harvested and 1/10 volume of 10 x Profinia protein A binding buffer was added, supernatant was filtered using an 0.45 nm filter membrane and used for protein A gel chomatography. ScFv-hIgG1Fc and scFv-mIgG2cFc antibodies were purified using standard BIO-RAD protocol for Profinia Protein A purification.

ScFv-mIgG1Fc antibodies were purified under high salt conditions adding NaCl to final 2 M to the cell culture supernatant and using a 1 x high salt binding buffer (2 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 8.1 mM KH_2PO_4).

5.11 Epitope mapping

Epitope mapping was performed on a cellulose acetate membrane onto which 15 amino acids long peptides with a three amino acid overlap were immobilized representing the complete amino acid sequence of the target protein human H5 A/Anhui/1/05 provided by Dr. Ronald Frank, HZI. Membrane was blocked in 2 % MPBST for 1 h at RT. A minimal amount of 10 µg scFv-mIgG1Fc antibodies wasincubated with the membrane in a maximum of 10 mL 2 % MPBST for 2.5 h at RT. After two washing steps in PBS for 5 min each the secondary antibody goat-α-Mouse IgG (Fc-specific) AP in a final dilution of 1:2,000 was incubated with the membrane in 10 mL 2 % MPBST for 1.5 h at RT. The membrane was washed twice 5 min each with TBST 0.05 % and twice 10 min each with CBS. Detection was performed using 10 mL staining solution consisting of CBS supplemented with 50 µL

1 M $MgCl_2$, 40 µL BCIP and 60 mL MTT (5 mg/mL). Incubation was performed over night protected from light. Membrane was washed twice with PBS before scanning. Membrane was stripped to remove staining and bound antibodies according to the following procedure with each washing step performed for 5 -10 min: 3 x H_2O, 2 x DMF, 3 x Stripping buffer A, 3 x Stripping buffer B, 3 x 100 % ethanol. After stripping membrane was stored at -20 °C.

5.12 Protein structure modelling

Hemagglutinin H5 protein sequence was derived from RCBS protein database (PDB accession number 2FK0[186] and protein structure was modelled using iMol.

5.13 Transmission electron microscopy

Sample preparatation and transmission electron microscopy was carried out by Prof. Dr. Manfred Rohde at the Helmholtz Center for Infection (HZI), Braunschweig. Thin carbon support films, approximately 10 nm thick, were prepared by sublimation of carbon from a carbon thread on to freshly cleaved mica strip. Using 300 mesh nickel grids virus samples were negatively stained with 4% (w/v) aqueous uranyl acetate[187]. Antibodies were directly labeled with 15 nm protein-A-gold according to manufacturer's procedures. Antibody labeled virus samples were stained by placing a butvar-coated 300 mesh copper grid for 30 sec on a drop of the virus suspension, washed in TE buffer and then negatively stained with a 2 % (w/v) aqueous uranyl acetate solution. Negatively stained samples were examined in a TEM with an acceleration voltage of 80 kV and at calibrated magnifications. Images were recorded digitally with a camera and respective software. For field emission scanning electron microscopy samples were adsorbed on to butvar-coated 300 mesh copper grids by placing a grid for 30 sec on a drop of the antibody labelled virus suspension, washed in TE-buffer, washed in H_2O and subsequentely air-dried. Grids were mounted on to conductive adhesive tabs on aluminum stubs. Samples were examined in a field emission scanning electron microscope using the Everhart-Thornley SE-detector and the inlens SE-detector in a 75:25 ratio at an acceleration voltage of 3 kV.

6 Results

6.1 Isolation of human anti H5 specific antibodies

6.1.1 Immobilization of recombinant human H5

To check for optimal immobilization conditions of recombinant human H5 A/Anhui/1/2005 target, various surfaces were used for initial immoblilization assays. Since conformational stability of the H5 molecule concerning pH value and temperature was yet unknown, different conditions were tested before pannings.

Three different immobilization strategies were analysed: ELISA plates and magnetic beads with either carboxylic acid groups or epoxylic acid groups on their surface (Fig 13). Best immobilization conditions were obtained for immobilization to carboxylic beads using NHS/EDC coupling and 10 mM sodium acetate buffer pH5.0 with complete coupling of the target, indicated by the loss of protein band after coupling. Immobilization efficiency to ELISA plates and Epoxy beads – irrespective of immobilization buffer – was comparable but less efficient for all three coupling procedures compared to immobilization to carboxylic bead.

Figure 13: Immobilization of recombinant human H5 A/Anhui/1/2005 to various surfaces.
500 ng of recombinant human H5 was immobilized to ELISA plates, carboxylic beads and Epoxy beads. 1/10 of the immobilization volume before and after immobilization was separated on a 10 % polyacrylamide gel and silver stained.

Immobilization conditions for human H5 antigen that were applied for pannings are summarized in Fig 14.

6.1.2 Pannings using human naïve antibody libraries

Naïve human scFv antibody phage-libraries HAL7 and HAL4 were constructed from non-immunized donors by Dr. Michael Hust and Saskia Helmsing[188] and kindly provided for this work. Four different independent panning campaigns were performed using a pool of two different naïve human antibody libraries, HAL7 and HAL4. Two panning campaigns were performed on Epoxy beads using two different immobilization buffers. Third and fourth panning campaign were performed on carboxylic beads and ELISA plates, respectively.

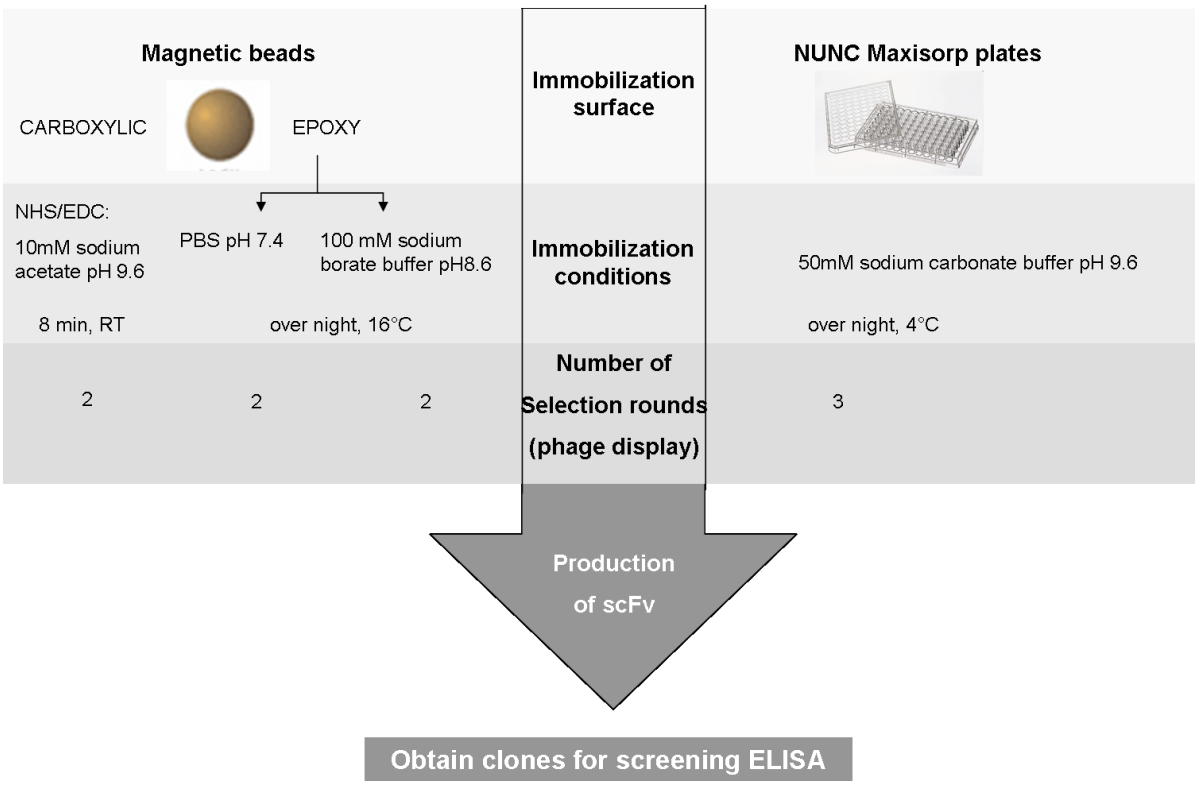

Figure 14: Panning strategy on human H5 A/Anhui/1/2005 with human naïve antibody libraries HAL7 and HAL4

After two successive rounds of pannings for all bead based pannings and three panning rounds for plate based pannings no enrichment of phage numbers could be retainably observed (data not shown). Screening ELISA was performed for some clones derived from third round of panning but no signal-noise ratio of 2:1 or better was obtained.

Since no H5 specific antibodies could be obtained from naïve human antibody libraries a competitve approach using H5 immunized chickens to construckt chicken immune libraries and select for H5 specific chicken antibodies was performed.

6.2 Generation of chicken immune libraries

Total RNA from immunized chickens from peripheral blood lymphocytes and spleen was isolated by the group of Prof. Dr. Timm Harder at the FLI according to their procedures using RNeasy RNA isolation Kit (Qiagen).

Nine samples (1-9) derived from blood samples (one double of one chicken for unknown reasons) and four samples (10-13) from spleen were used for library construction.

6.2.1 Amplification of chicken antibody genes

Generated cDNA was used for amplification of chicken antibody genes via PCR and a gel band of a size of about 400 bp was excised after separation on an agarose gel.

VH and VL genes were amplified from all samples although DNA yield for samples 4 and 6 for both antibody chains were very low (data not shown). DNA obtained from the first PCR amplification was used for a second PCR to introduce restriction sites to facilitate cloning into antibody phage display vectors. For all thirteen samples fragments with correct size of about 400 bp were amplified and excised from agarose gel (Fig 15).

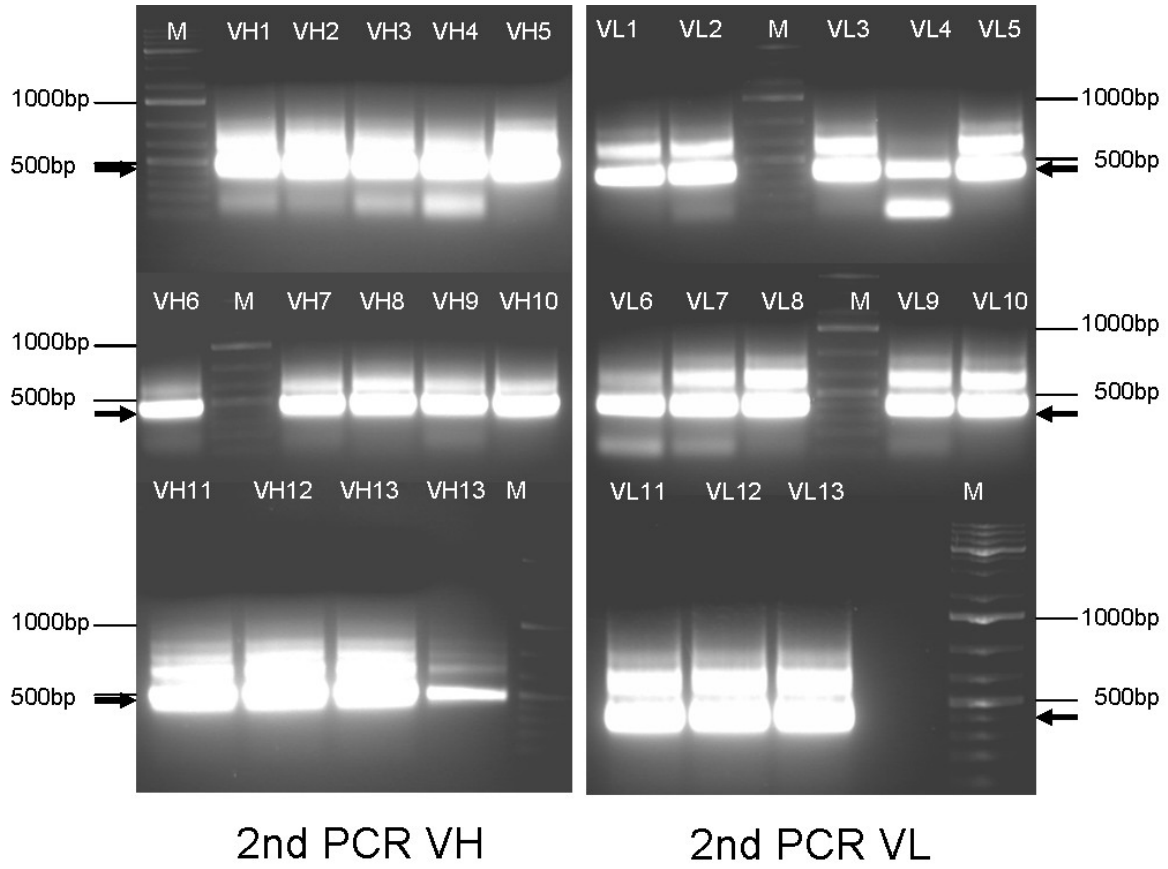

Figure 15: Amplification from chicken antibody genes to introduce cleavage sites.
Numbering of VH/VL is according to blood (1-9) or spleen (10-13) samples, respectively. Arrows indicate bands excised from gel corresponding with chicken antibody VL/VH.

Differences in DNA amount observed after first PCR round were reduced after second PCR round and for all 13 samples sufficient DNA amounts were obtained to be cloned into phage display vector pHAL 14.

6.2.2 Packaging of chicken antibody genes into phage libraries

After restiction digest with appropriate restriction enzyme fragments were cloned into the pHAL 14 antibody phage display vector. Due to observed restriction for *Mlu*I of chicken VL and *Nco*I of chicken VH antibody genes (data not shown) the cloning strategy had to be modified to reduce negative effects on antibody library diversity.

VH antibody gene fragments were cloned into pHAL14 first followed by introduction of VL antibody gene fragments into phage display vector pHAL 14 already containing VH fragments.

After transformation pHAL 14 vectors containing VH and VL antibody fragments in *E. coli* XL1 blue a total amount of 5.7×10^6 cfu and 1.6×10^7 cfu independent clones were obtained derived from blood and spleen, respectively.

6.2.3 Colony PCR of clones from packaged chicken immune libraries

Quality of constructed chicken anti-H5 immune libraries was analysed regarding numbers of independent clones obtained after transformation of pHAL14 phage display vector containing both antibody chains (VH and VL), percentage of incorporated full-size single-chain antibody gene fragments, phage titres of final chicken immune libraries after packaging of phagemid DNA into phages and fusion of single-chain antibodies on phage pIII-surface protein.

Antibody fragments of 29 clones were amplified as a reperesentative for each chicken library. Concerning blood derived chicken library KFC2_AIV-B about 90 % of the analysed clones contained full-length antibody gene fragments of about 1100 bp. With regard to spleen derived library KFC2_AIV-S percentage of full-length inserts scored 100 % (Fig. 16)

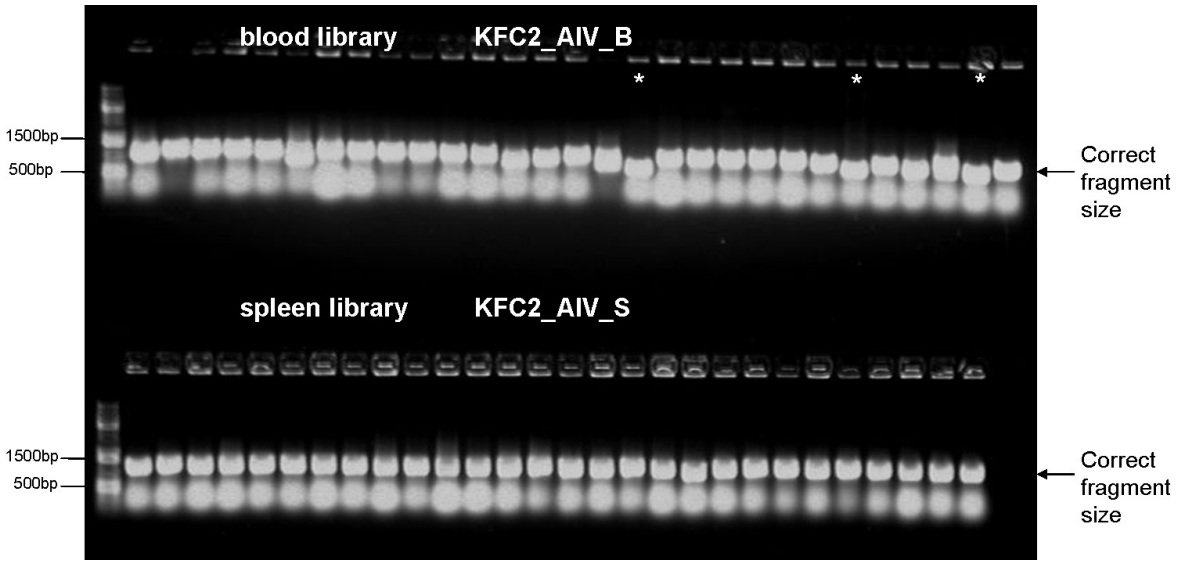

Figure 16: Colony PCR of clones from packaged chicken immune libraries
ScFv-antibody fragments were amplified using pHAL14 vector specific oligonucleotides and separated on a 1% agarose gel. Correct full length scFv-antibody fragments show a band at ~ 900bp, clones missing one or both variable regions have bands of less than 900 bp and are indicated by *.

6.2.4 Anti-pIII immunoblot

After packaging of phagemid DNA into phages the following phage titres were obtained for both chicken anti-H5 immune libraries: for KFC2_AIV-B $1.3x10^{14}$ cfu/mL and for KFC2_AIV-S $9.4x10^{13}$ cfu/mL. Antibody phage preparations were analysed for the presentation of scFv-antibodies on the surface of the phage. A clear higher apparent fragment size of the pIII-protein of the phage preparations compared to wild-type pIII from helper phage was observed in the anti-pIII immunoblot (Fig 17). This difference in size indicates the fusion of the scFv antibody to the phage coat

protein pIII.

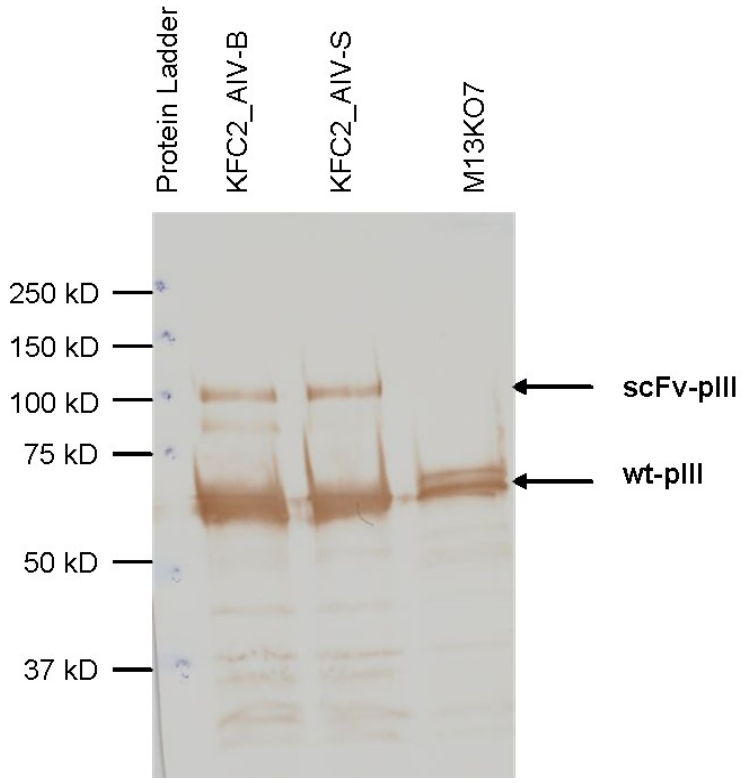

Figure 17: Immunoblot of phage packaged chicken immune libraries
Packaged phage preparations from blood (KFC2-AIV-B) and spleen (KFC2-AIV-S) derived chicken antibody phage libraries were separated under denaturing conditions on a polyacrylamide gel and blotted onto a PVDF-membrane. Detection was performed using an anti-pIII-antibody and a HRP-labeled goat-anti-mouse-Fc specific antibody. Staining was performed using DAB substrate. M13KO7 was used as reference of wildtype pIII (wt-pIII). Protein ladder was from Bio Rad.

Generated chicken anti-H5 immune libraries were used for pannings on two different H5 antigens.

6.3 Isolation of chicken anti-H5 antibodies

To direct selection pressure towards the isolation of antibodies that broadly detect H5, two different recombinant H5 protein targets from different origins were introduced into the panning campaign: H5 from human origin (A/Anhui/1/2005) and H5 from avian origin (A/Canada goose/Germany/71/05). Pannings and screenings were performed separately on both targets. Only those antibodies that bound to both H5 antigens were further analysed. A summary of this procedure is given in (Fig 12).Commercial recombinant human H5 A/Anhui/1/2005 protein was immobilized to magnetic beads using 10 mM sodium acetate buffer, pH 5.0. The biotinylated recombinant H5 A/Canada goose/Germany/71/06 provided by the group of Prof. Timm Harder at the FLI was produced as trimer [184] and captured using streptavidin

magnetic beads.

6.3.1 Pannings using chicken immune libraries

Panning campaigns applied for the identification of chicken H5-specific antibodies that broadly bind to H5 proteins from different origins are summarized in (Fig 12).

Three successive rounds of pannings were performed on human recombinant H5 antigen. Phage titre was stable from first to second round but showed from round two to three a more than 30 fold increase for KFC2_AIV-B and a nearly 100 fold fold increase for KFC2_AIV-S (Fig 38). Two successive rounds of pannings were performed on avian recombinant trimeric H5 antigen. Phage titre was stable from first to second round (Fig 39).

To maintain the achieved diversity of bindersscreening ELISA were performed for clones derived from panning round two for both panning strategies.

6.3.2 Screening for chicken scFv antibodies

From the second round of panning 94 clones were screened for positive binders for each target and each library separately. Screening results are summarized in Tab 11.

Table 11: Summary of screening ELISA on human H5 and avian H5 using scFv clones from chicken immune antibody libraries

Chicken Immune Library	Number of analysed clones	Number of scFv-antibody clones binding on		
		human H5	avian H5	human and avian H5
KFC2-AIV_B (blood)	94	82	67	67
KFC2-AIV_S (spleen)	94	81	72	71

Examples of screening results are given in Figs 18 and 19. Twelve scFv antibodies with positive signals on both panning targets were subjected to further analyses.

From 94 clones analysed on carboxy beads and to ELISA plates immoblized human H5 antigen more than 85% of all clones from each library showed positive signals on human H5 target. Signal was considered to be positive with a signal/noise ratio of 5:1 or better at $OD_{450\ nm}$. In contrast, about 65% of all clones from pannings on avian H5 antigen showed positive signals on the respective target captured via streptavidin magnetic beads from protein expression supernatant. The vast majority of human H5 selected antibodies bound to plate immobilized-H5 as well as to bead immobilized-H5. Some binders which showed positive signals on plate immobilized H5 but very low values on bead immobilized H5 were considered to be negative. Examples of screening data for both chicken antibody libraries KFC2_AIV-B and KFC2_AIV-S are

shown in Fig 18. and Fig 19 for the human and avian H5 target, respectively.

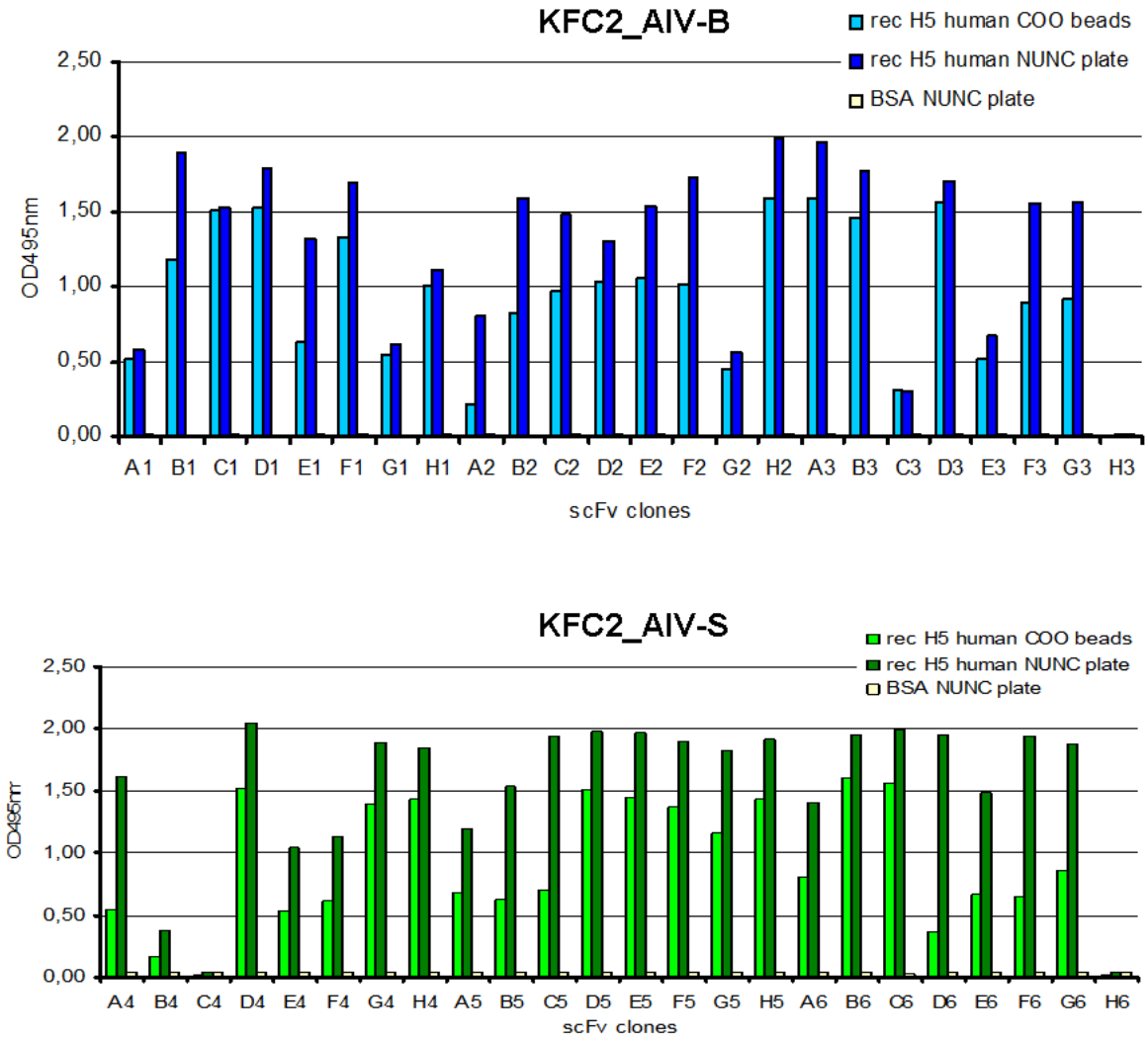

Figure 18: Screening ELISA with chicken scFv clones obtained from pannings on recombinant human H5 A/Anhui/1/2005 (examples).
ScFv-clones originating from pannings using chicken immune libraries constructed either from blood RNA (KFC2_AIV-B) or from spleen RNA (KFC2_AIV-S). Binding properties were analysed on recombinant H5 A/Anhui/1/2005 immobilized on carboxylic beads, on ELISA plates and on BSA.

Overall signals on avian H5 antigen were relatively low compared to screening results on human H5 (Figs 18, 19) due to the assumed low amount of avian H5 immobilized to streptavidin bead surface. As avian H5 protein from cell culture supernatant could only be detected in ECL stained immunoblot (Fig 21) H5 protein amount in the supernatant is assumed to be low. Furthermore, only a small amount of baculoviral expression supernatant was used for H5 immobilization to streptavidin beads with respect to the limited amount of avian H5 cell culture supernatant that was available for these analyses. All antibodies that bound avian H5 in screening ELISA also bound to human H5.

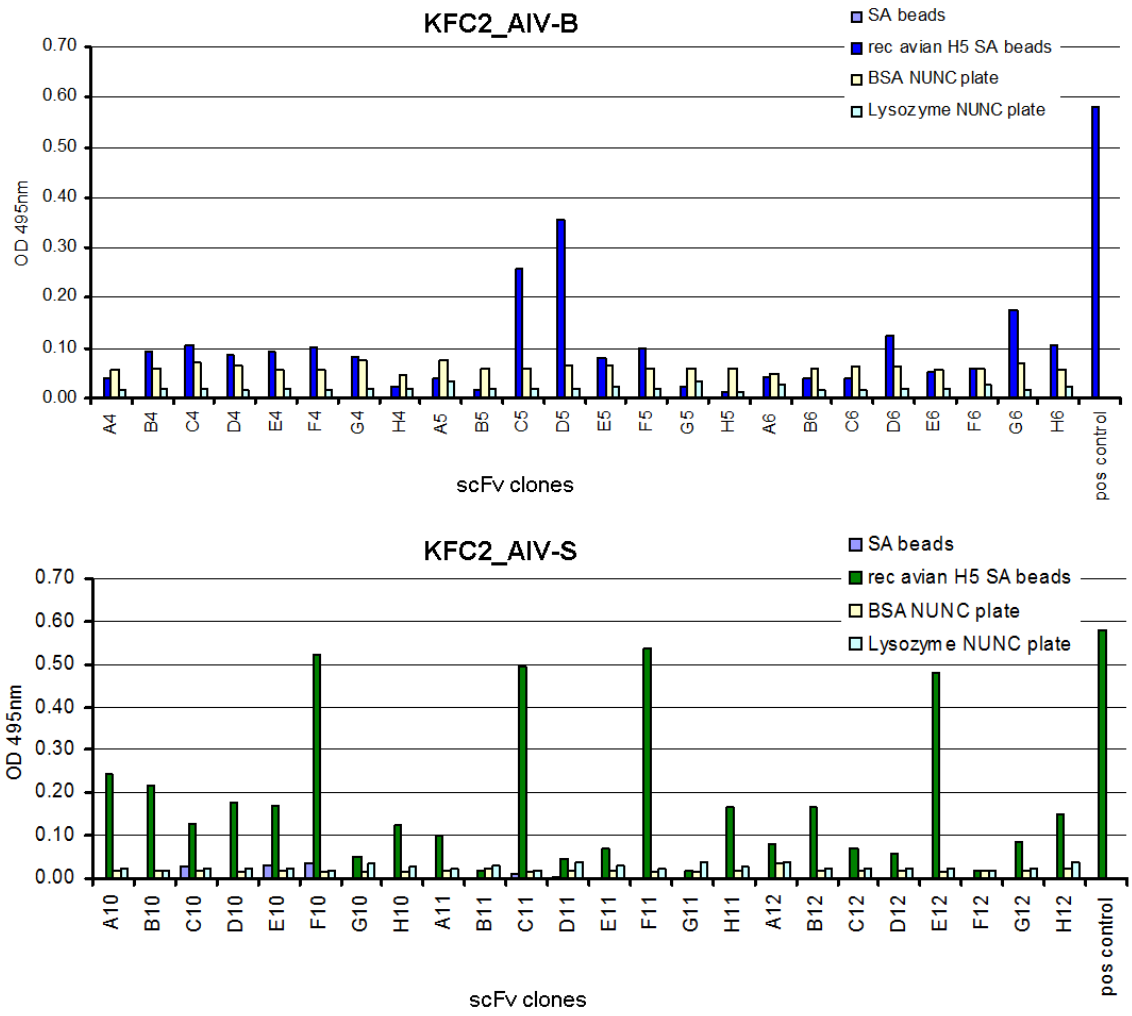

Figure 19: Screening ELISA with chicken scFv clones obtained from pannings on avian recombinant H5 A/Canada goose /Germany/71/06(examples).
ScFv-clones originating from pannings using chicken immune libraries constructed either from blood RNA (A: KFC2_AIV-B) or from spleen RNA (B: KFC2_AIV-S) were analysed for binding on recombinant H5 A/Canada goose/Germany/71/06 immobilized to Streptavidin beads, and for negative control on empty Streptavidin (SA) beads, BSA and lysozyme – both immobilized on ELISA plates. A total of 10 µL baculoviral expression supernatant was immobilized to 1µL streptavidin beads and used per clone.

6.4 Sequence analysis of isolated chicken antibodies

All positive clones derived from screening ELISA were subjected to *Bst*NI-digest to perform DNA fingerprinting (data not shown). A total of 30 individual clones were sequenced and revealed twelve different scFv antibodies (Fig 39). in the appendix).

Up to five or eight amino acids have been inserted into CDR3 regions of VL or VH chains, respectively, compared to germline VH and VL sequences, in these twelve clones. Furthermore, genetic variability was observed in all CDRs. Six of the antibodies isolated (JM7_1, _2, _4, _10, _11, _13) share the same VH chain except for singe amino acid subtitutions in conserved regions but show major differences in their respective light chains. Antibodies JM03 and JM06 show major mutations in

CDR1 of VL chain. Both show the same insertion of five amino acids YAGSYY) in this region. Three hot spot regions of accumulated mutations occur between VL CDR 2 and CDR 3, one at positions 220, 230 and 236.

6.5 Cloning, production and purification of chicken scFv-Fc antibodies

All twelve scFv antibodies JM7_01 to JM7-11 were cloned into expression vectors allowing the expression of scFc-Fc antibodies in mammalian cells with two scFv antibodies fused to an Fc-part of an antibody of either human or murine origin.

All antibodies were cloned into pCMX2.5-hIgG1Fc and pCMV2.5-mIgG1Fc expression vectors (Figs 44, 45) allowing the expression of scFv-hIgG1Fc and scFv-mIgG1Fc, respectively. Both variants of the scFv-Fc antibody format were expressed in adherent HEK293T cells.

After the observation of instability of scFv-mIgG1Fc antibodies in SDS-PAGE analyses (data not shown) all twelve unique anti-H5 antibody clones were cloned into pCSE2.5-mIgG2cFc vector (Fig 43) allowing the expression of murine IgG2cFc and expressed in HEK293-6E cells cultures.

6.6 Analyses of scFv-Fc antibodies

6.6.1 Immunoblots

For further investigation of antibody binding properties immunoblot with various H5 samples, recombinant proteins and virus samples, and H7 for control was performed. For all immunoblots scFv-hIgG1Fc constructs were used. Influenza hemagglutinin shows a band at about 72 kD of apparent protein size on SDS-PAGE and in immunoblots

All antibodies showed binding to recombinant panning target human H5 A/Anhui/1/2005 (Fig 20) and except for antibody JM7_7 to recombinant panning target avian H5 A/Canada goose/Germany/71/05 as well (Fig 21).

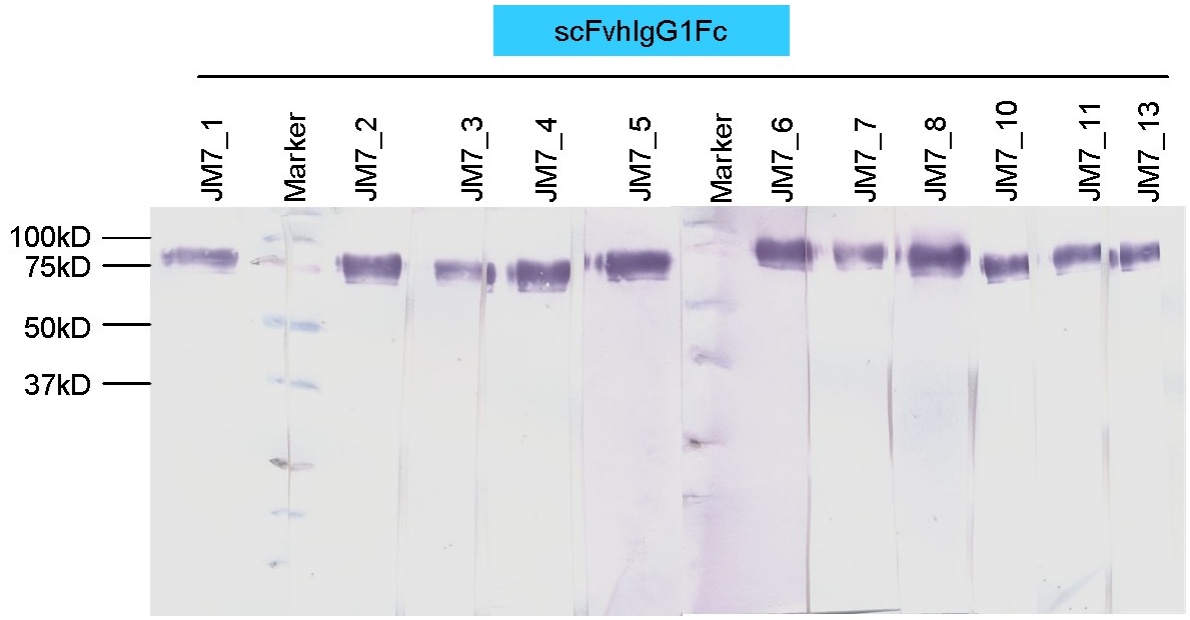

Figure 20: Anti-human H5 A/Anhui/1/2005 immunoblot using scFv-hIgG1Fc antibodies.
Recombinant target was separated on a 10 % SDS polyacrylamide gel and blotted onto PVDF membrane. Bound scFv-hIgG1Fc antibodies were detected using and HRP labeled anti-human-Fc-antibody and TMB solution for staining.

In Fig 21 clones JM7_3, JM7_9 and JM7_12 are idividual clones with identical amino-acid sequence as well as clones JM7_13 and JM7_14.

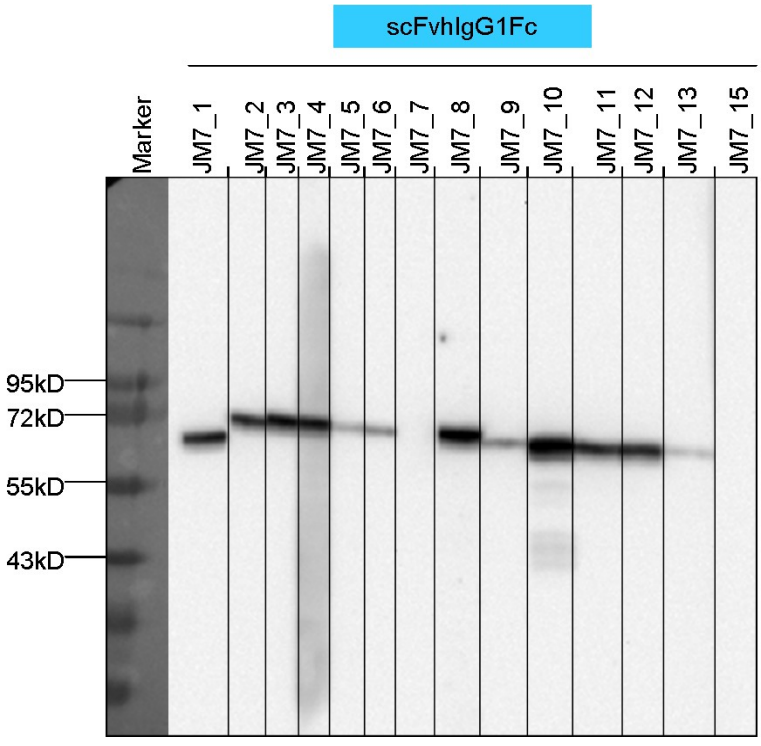

Figure 21: Anti-avian H5 A/Canada goose/Germany/71/06 immunoblot using scFv-hIgG1Fc antibodies.
Baculovirus expression supernatant was separated o a 10 % SDS polyacrylamide gel and blotted onto a PVDF mebrane. Bound scFv-hIgG1Fc antibodies were detected via chemiluminescence using and HRP labeled anti-human-Fc-antibody and ECL-detection kit.

To further analyse whether antibodies bind to the head like structure HA1 of influenza hemagglutinin or to the stem like structure HA2, samples containing whole influenza viruses produced in embryonated chicken eggs were used for immunoblots (Fig 22, 23). Upon protease cleavage during processing of influenza viruses in ECE unprocessed HA0 protein is cleaved into subunits HA1 (distal part of the hemagglutinin comprising knob like head of the protein and HA2 (membrane associated part of the hemagglutinin molecule). These three different hemagglutinin fractions HA0, HA1 and HA2 clearly differ in apparent molecular size in SDS-PAGE and immunoblot (HA0 ~ 72 kD, HA1 ~ 50 kD and HA2 ~ 26 kD) and antibody binding to either HA1 or HA2 subunit can be determined in immunoblots using processed influenza viruses. Three different H5 influenza virus strains were used for immunoblots (Figs 22, 23). and the H7 influenza virus in position 2 was chosen to control subtype specificity.

Two sera from chickens immunized with H7 avian influenza virus (S87) and with H5 avian influenza virus (S89) are used in immunoblot for reference (Fig 22). The anti-H7 serum S87binds to HA0, HA1 and HA2 of influenza hemagglutinin and shows cross reactivity with H5 A/teal/Föhr/Wv632/05(lane 3) and H5 A/Vietnam/1197/2004 (lane 4). S89 is known to be a “monoclonal” anti-H5 serum and binds the HA2 subunit of hemagglutinin [184]. Nevertheless, it also shows binding to HA1 of H5A/teal/Föhr/Wv632/05 (lane 3), although HA1 shows unusual apparent size in this lane.

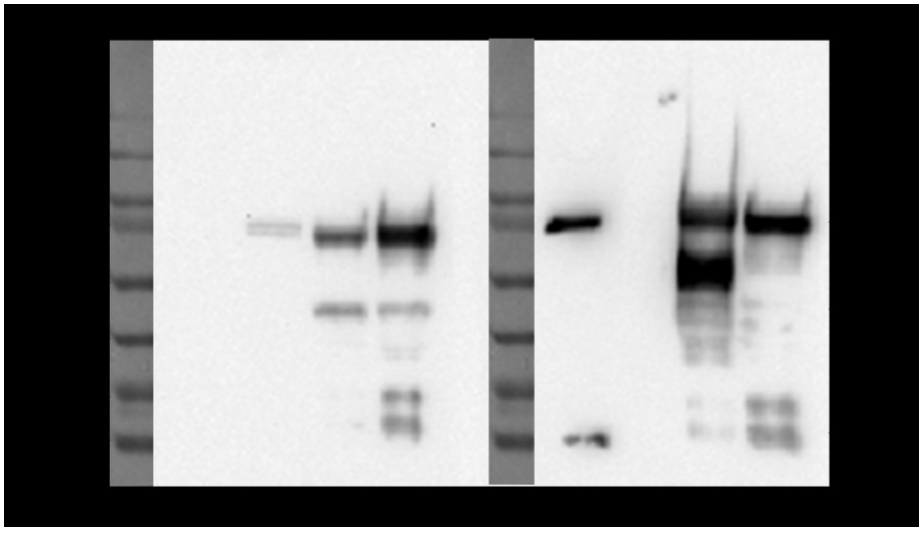

Figure 22: Immunoblot of two different chicken anti-influenza hemagglutinin sera on different hemagglutinin targets
Lane 1: H5 A/Canada goose/Germany/71/06, lane 2: H7 A/chicken/Germany/R28/03, lane3: H5 A/teal/Föhr/Wv632/05, lane 4: H5 A/Vietnam/1197/2004; M: protein marker peqGold IV; HA0:

uncleaved precursor hemagglutinin, HA1: globular head domain of hemagglutinin, HA2: stalk domain of hemagglutinin; staining: chemiluminescence

None of the antibodies bound to hemagglutinin H7 (lane 2) and theH5 A/Vietnam/1197/2004 (lane 4) was difficult to detect or even undetectable. No antibody bound the HA2 fragment, thus all antibodies bind to HA1 subunit, the head like part of the hemagglutinin molecule. Antibodies JM7_15 and JM7_7 detected only unprocessed HA0 of H5 A/Canada goose/Germany/71/06 (lane1) but showed binding to cleaved HA1 of H5A/teal/Föhr/Wv632/05 (lane 3). Therefore, these two antibodies are considered to bind to HA1 part of influenza hemagglutinin as well.

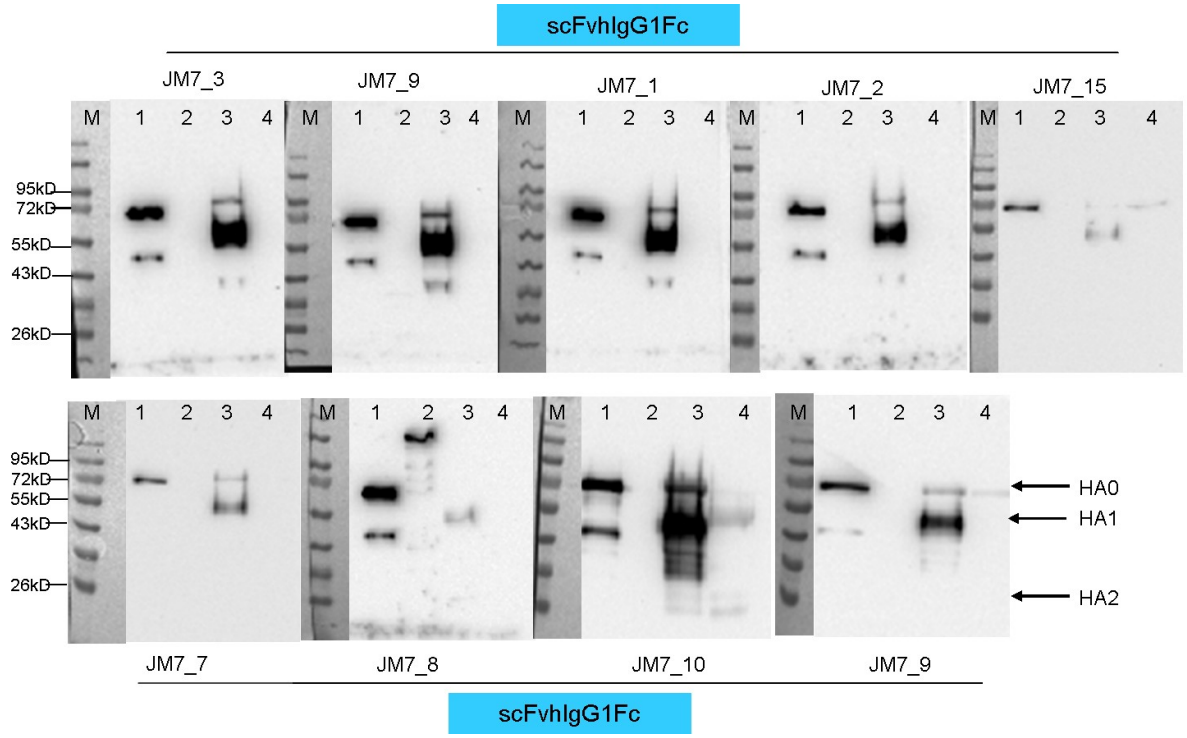

Figure 23: Immunoblot of different scFv-hIgG1Fc antibodies on different hemagglutinin targets. Virus samples were separated on a 10 % SDS polyacrylamide gel and blotted onto a PVDF mebrane. Lane 1: H5 A/Canada goose/Germany/71/06, lane 2: H7 A/chicken/Germany/R28/03, lane3: H5 A/teal/Föhr/Wv632/05, lane 4: H5 A/Vietnam/1197/2004. Bound scFv-hIgG1Fc antibodies were detected via chemiluminescence using and HRP labeled anti-human-Fc-antibody and ECL-detection kit. M: protein marker peqGold IV; HA0: uncleaved precursor hemagglutinin, HA1: globular head domain of hemagglutinin, HA2: stalk domain of hemagglutinin, staining: chemiluminescence

All selected antibodies bind to the head like structure of influenza hemagglutinin since exclusively unprocessed HA0 and HA1 hemagglutinin were bound in immunoblots.

To further analyse thedetection of recently emerged highly variable escape mutants Egyptian H5N1 virus strain A/chicken/Egypt/0879-NLQP/2008-R737/09 was chosen for immunoblot. This virus has accumulated many mutations within the globular head of the hemagglutinin and thus effectively escapes from immune response [120,122,189].

Two o#f the isolated anti-H5 antibodies, JM7_6 and JM7_8, detected this Egyptian escape mutant in immunoblots (Fig 24).

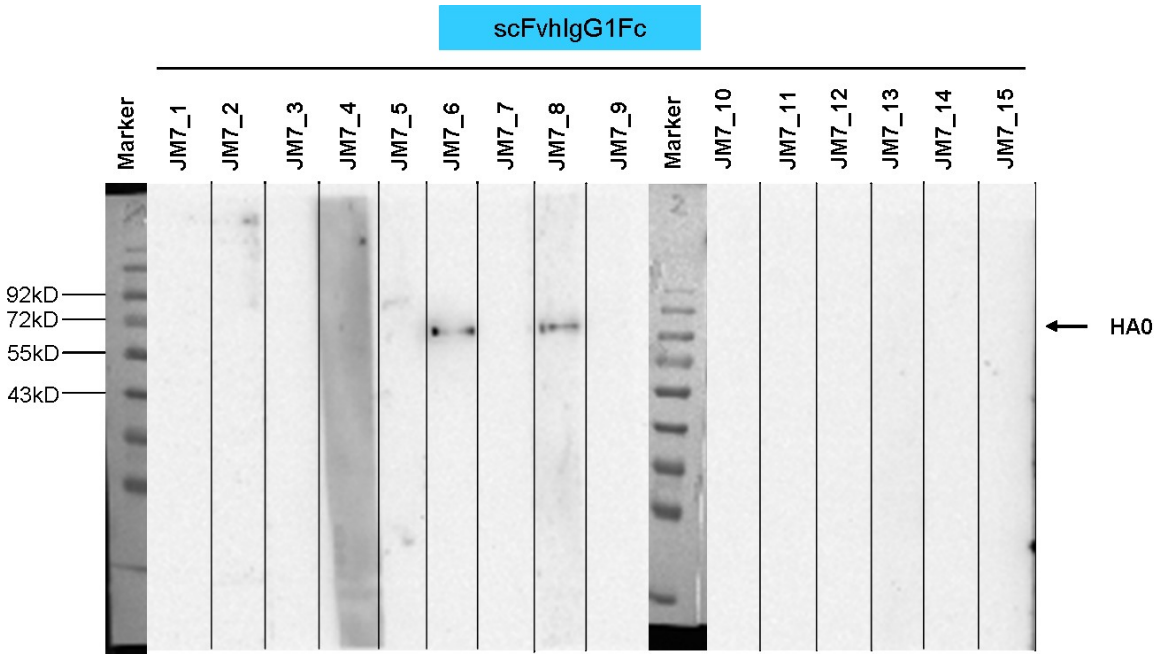

Figure 24: Immunoblot on inactivated virus A/chicken/Egypt/0879-NLQP/2008-R737/09 using scFv-hIgG1Fc antibodies.
Marker: protein marker peqGold IV, staining: chemiluminescence

Antibody clones HJM7_6 and JM7_8 bound H5 to A/chicken/Egypt/0879-NLQP/2008-R737/09.

6.6.2 Detection of human and avian H5 antigen in ELISA using scFv-hIgG1Fc antibodies

After expression and purification of scFv-hIgG1Fc antibodies these constructs were analysed on both panning targets again to demonstrate conservation of binding properties regarding these two targets (Fig 25). Values represent the means of absorbances of duplicate wells.

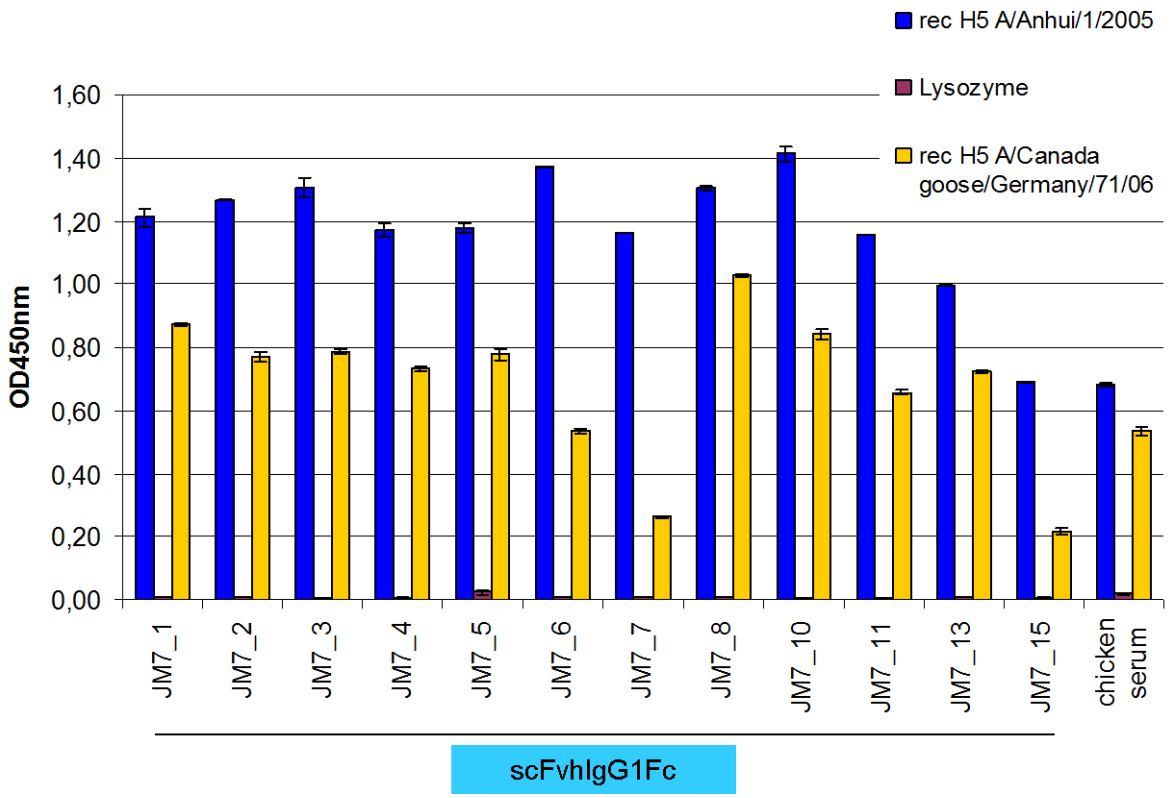

Figure 25: Binding of scFv-hIgG1Fc antibodies to recombinant human H5 and recombinant avian H5 A/Canada goose/Germany/71/06
A total of 125 ng recombinant human H5A/Anhui/1/05 and lysozyme (for negative control) was immobilized per well to ELISA plates. Recombinant avian H5 A/Canada goose/Germany/71/06 was captured from baculoviral expression supernatant using streptavidin magnetic beads. A total of 500 ng scFv-hIgG1Fc antibodies was used per well with 200 µL supernatant per 1 µL beads and target bound antibodies was detected using HRP conjugated goat-anti-human-Fc antibody.

6.6.3 Determination of antibody affinities and detection limits

To determine minimal detectable amount of human recombinant H5 an antigen titration ELISA was perfomed and revealed that at least 5 ng per well were detectable with an absorption value of about 0.4 for all antibodies. (Fig. 40). Values represent mean absorbances of duplicate wells.

A minimal amount of 1 - 5 ng scFv-hIgG1Fc antibody was necessary to obtain positive signals in antibody titration ELISA using 50 ng human H5 at an absorption value of 0.4-0.6 (Fig 41). Values represent mean absorbances of duplicate wells.

Based on the molecular weight of scFv-Fc antibodies of ~ 104 kDa, the antibody binding curves obtained from antibody titration assay (Fig 41) with the indicated antibody concentrations the half maximal absorption values (EC_{50}) of several scFv-hIgG1Fc antibodies were calculated (Tab 12). All antibodies bound the human H5 antigen in a subnanomolar range with the clone JM7_1 showing an EC_{50} of 0.14 nmol/l.

Table 12: EC 50 values of scFv-hIgG1Fc antibodies

scFv-hIgG1Fc clone	JM7_1	JM7_2	JM7_3	JM7_4	JM7_8
EC_{50} [M]	$1.4x10^{-10}$	$6.7x10^{-10}$	$1.9x10^{-10}$	$7.7x10^{-10}$	$3.8x10^{-10}$

6.6.4 Detection of H5 hemagglutinin in virus samples using capture ELISA

In order to analyse epitope binding sites of the individual antibodies and to demonstrate their use in detection systems, capture ELISA and membrane based capture immunodot assays were performed.

In capture ELISA both panning targets could be detected by different pairs of the selected antibodies (Figs 26 and 27). Signals for recombinant human H5 were higher compared to recombinant avian H5 since avian H5 of unknown concentration had to be captured from baculoviral expression supernatant whereas human H5 was used in a defined amount of 75 ng per well. For control and to analyse possible unspecific binding of immobilized scFv-h-IgG1Fc and detecting scFv-mouse IgG1Fc wells were incubated in 1% MPBST as blocking solution. Unspecific interaction of the two antibody formats was observed for JM7_1 but not for JM7_8 and JM7_3 (Fig 26). Values represent mean absorbances of duplicate wells.

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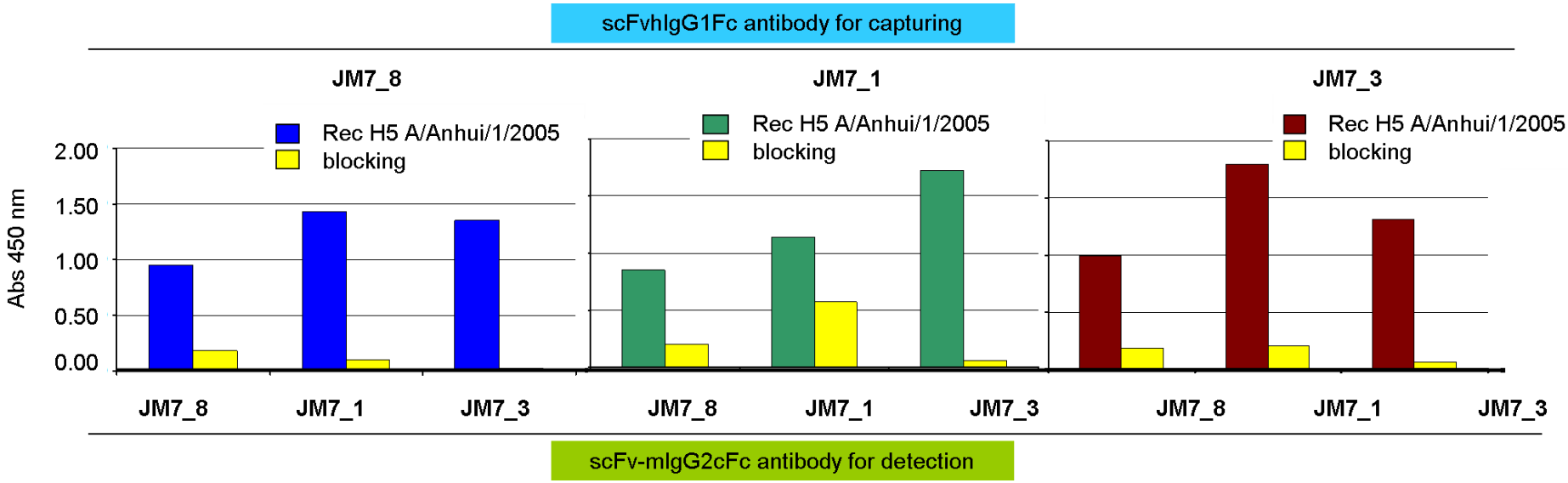


Figure 26: Capture ELISA using recombinant human H5 A/Anhui/1/2005.
500 ng of scFv-hIgG1Fc were immoblilized to ELISA plates per well. As a target 75 ng recombinant H5 A/Anhui/1/2005 was used. For detection 500 ng/well of scFv-mIgG2Fc and a HRP-labeled goat-anti-mouseFc antibody were used. As a negative control 1% MPBST was used instead of target (blocking). Staining was performed using TMB.

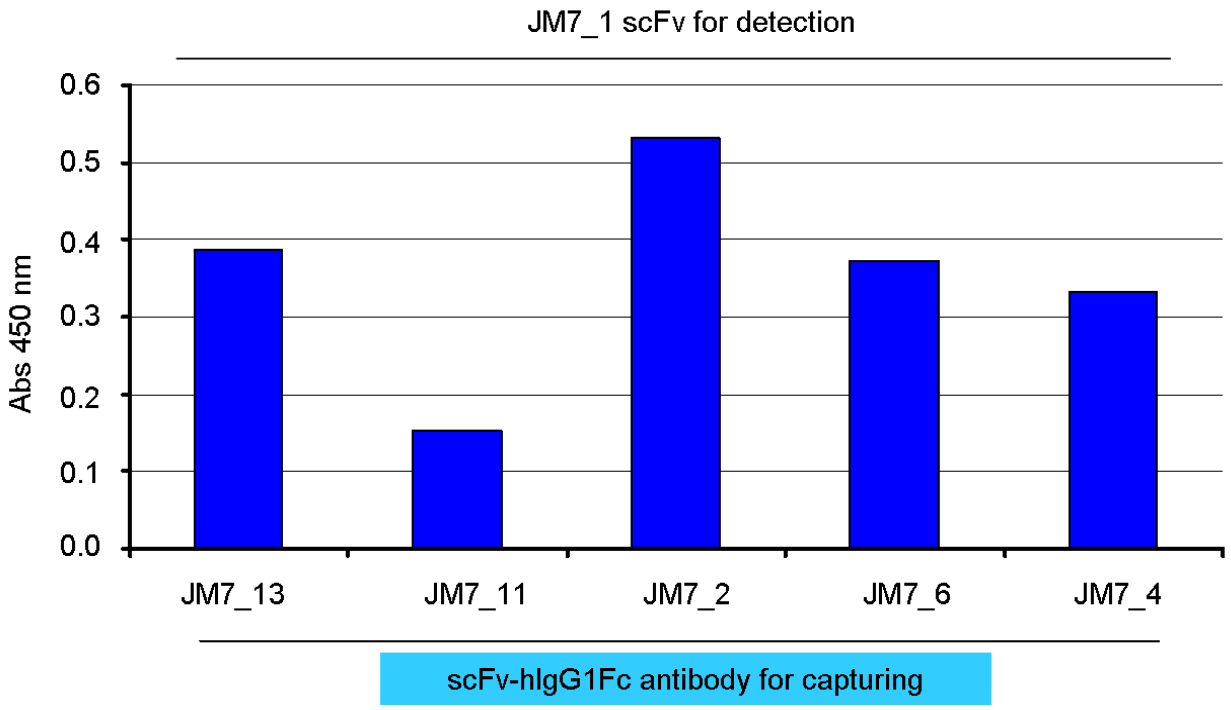


Figure 27: Capture ELISA using recombinant avian H5 A/Canada goose/Germany/71/06.
400 ng of scFv-hIgG1Fc antibodies were immoblilized to ELISA plates per well. As a target 40 µL baculovirus expression supernatant of recombinant trimeric H5 A/Canada goose/Germany/71/06 was used and detected using 50µL JM7_1scFv, mouse-anti-myc-antibody and HRP-labeled goat-anti-mouseFc antibody. Staining was performed using TMB.

Capturing of whole viruses from allantoic fluid preparations was also assayed (Fig 28). Viruses were propagated in embryonated chicken eggs. Initially all possible antibody combinations were analysed for capturing of H5N1 A/teal/FöhrWv632/05 virus preparation (data not shown). Antibody pairs successful in capturing A/teal/Föhr/Wv632/05 virus were applied in detailed capture ELISA using twelve different influenza virus preparations or antigens. The antibody combination JM7_1 scFv-hIgG1Fc and JM7_3 scFv-mIgG1Fc showed highest absorption signals and broadest virus detection spectrum restricted to H5 viruses. Low pathogenic influenzua viruses A/mallard/B.C./544/05 (H5N9) and A/goose/Manitoba/428/06 (H5N2) remained undetectable (Fig 28). Concerning the inactivated virus preparations of highly pathogenic influenza viruses only A/Canada goose/Germany/71/06, the origin of the trimeric avian recombinant antigen used in panning campaigns, was detected. Under mild denaturing conditions (preincubation of virus preparation in 1 % Tween 20 or 1 MPBS over night incubation at 4 °C detection of A/mallard/B.C./544/05 could be improved (data not shown). In contrast under modified conditions a significant reduction in detection signal was observed for A/teal/Föhr/Wv632/05 (data not shown).

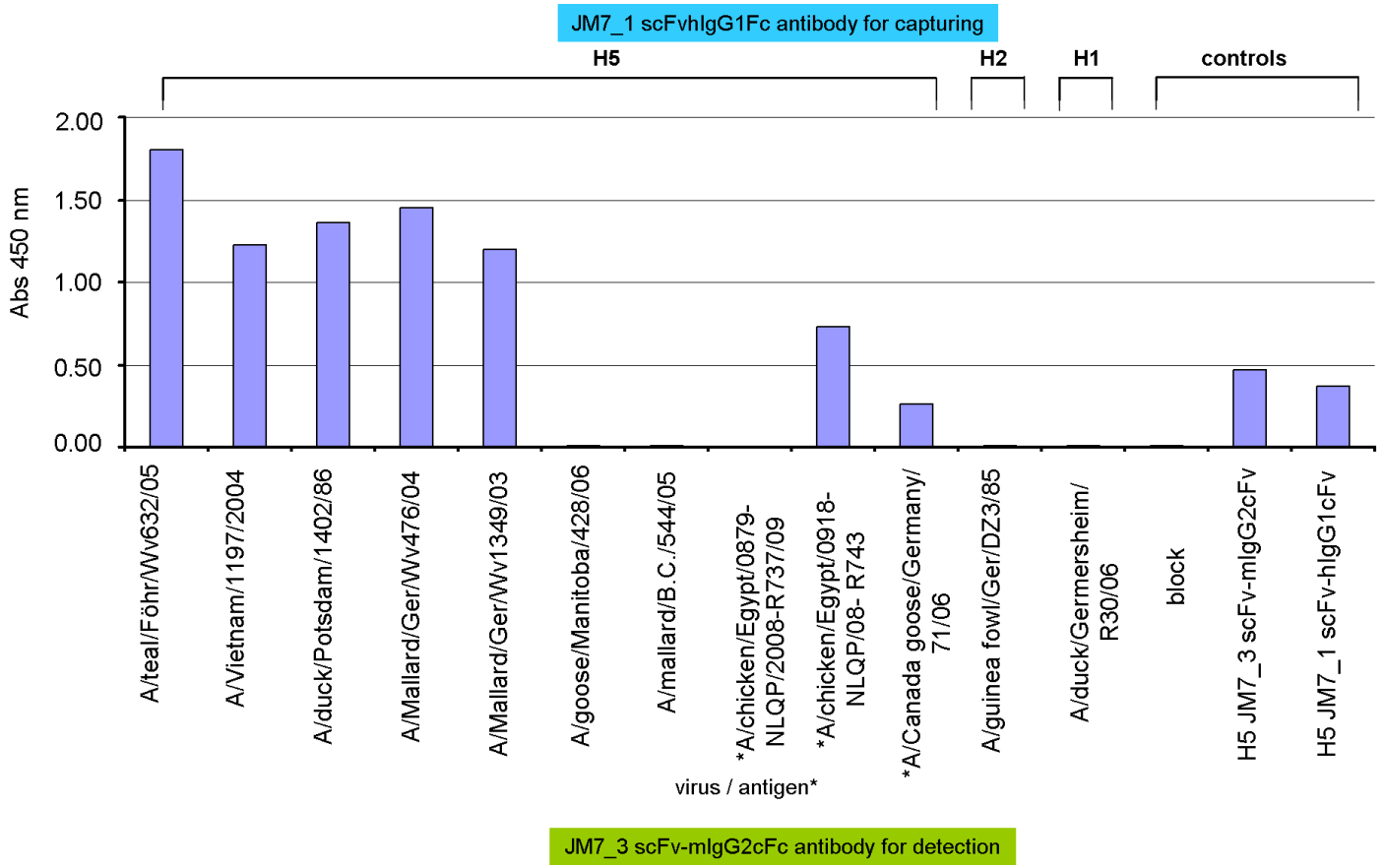


Figure 28: Capture ELISA using antibody pair JM7_1 scFv-hIgG1Fc/JM7_3 scFv-mIgG1Fc to detect different influenza viruses from allantoic fluid preparations

A total amount of 500 ng of JM7_1 scFv-hIgG1Fc antibodies was immobilized to capture virus from allantoic fluid preparations. Virus antigen was adjusted to a minimal HA titre of 8 except for A/Mallard/Ger/Wv476/04 (H5N2) (HA-titre 5), A/mallard/B.C./544/05 (H5N9) (HA-titre 4) and A/chicken/Egypt/0918-NLQP/08 H5N1 HP (R743) (HA-titre 6) due to low HA-titres of virus original preparation. A total amount of 500 ng JM7_3 scFv-mIgG1Fc antibody was used and HRP-conjugated goat-anti-mouseFc antibody secondary antibody. To control quality of capturing antibodies 100 ng of recombinant human H5 A/Anhui/1/2005 was immobilized and binding of 500 ng of the respective scFv-Fc antibody was analysed. For scFv-hIgG1Fc antibody an HRP-conjugated goat-anti-hu manFc antibody was used as secondary antibody. For negative control blocking solution instead of virus was used (indicated by 'block'). Staining was performed using TMB.

6.6.5 Detection of H5 hemagglutinn in virus samples using a membrane based immunodot blot

To show detection of whole viruses using a membrane based capturing system an immunodot blot was performed (Fig. 29) with an scFv-hIgG1-Fc antibody immobilized to a nitrocellulose membrane capturing the virus. ScFv-mIgG2-Fc antibody and an HRP labeled goat-anti-mouse Fc antibody were used for virus detection. Two different Fc-species were used to avoid crosss-reactivity of the labeled antibody with the immobilized capturing antibody. A 100 ng dot of scFv-hIgG1Fc antibody JM7_3 was sufficient to capture avian influenza virus A/teal/Föhr/Wv632/05 from allantoic fluid produced in embryonated chicken eggs.

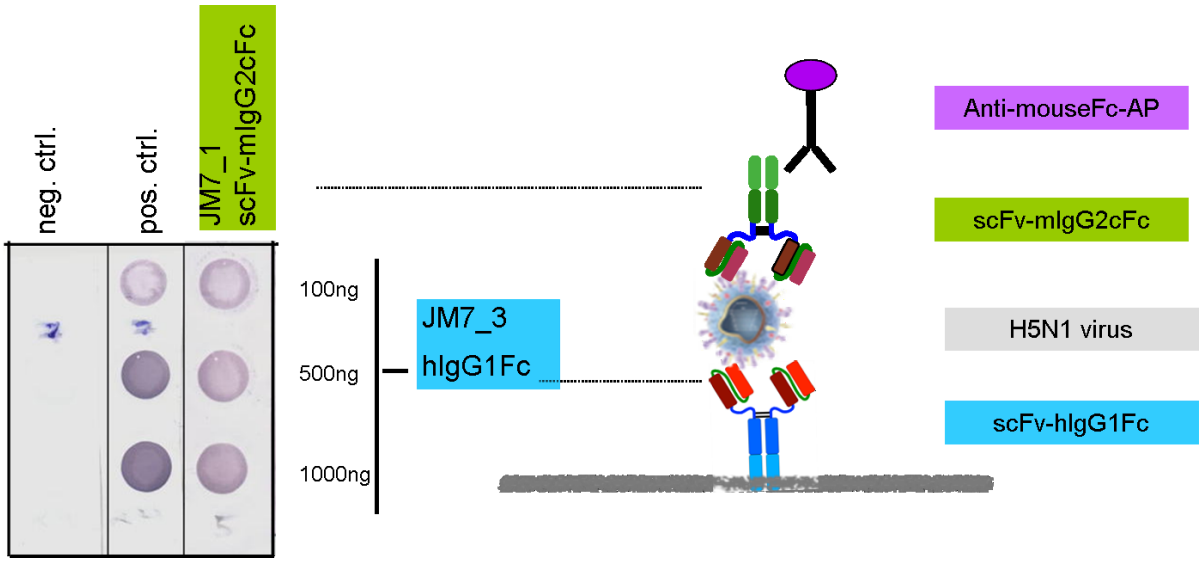


Figure 29: Capture Immunodot blot using influenza virus A/teal/Föhr/Wv632/05.
As an example 0.5mL supernatant of H5N1 avian influenza virus A/teal/Föhr/Wv632/05 produced in embryonated chicken eggs was used. Indicated amounts of JM7_03 scFv-hIgG1Fc antibody was immobilized and soluble JM7_1 mIgG2cFc antibody [2.5µg/mL] and alkaline phosphatase labeled goat-anti-mouse Fc- antibody was used for detection. Staining was performed using NBT/BCIP.

6.6.6 Immunoperoxidase monolayer assay (IPMA)

To further analyse H5 specificity of the isolated antibodies an ELISA on influenza virus infected cells was performed. A set of nine different low pathogenic avian influenza viruses of the H5 subtype and three non-H5 viruses (H1, H2, H6) were used to infect a swine testis cell line. Viral proteins are expressed in the cytoplasm and on the cell surface upon infection and can be detected by antibodies after fixation of the cells. To analyse antibody binding to highly pathogenic influenza viruses recombinant insect cells, which stably express viral H5 on their surface, were used. ScFv-hIgG1Fc antibodies were used throughout this assay. Tab 13 summarizes the obtained results.

Table 13: Overview of IPMA results for all scFc-hIgG1Fc antibodies.

			scFv-hIgG1Fc antibodies JM7_											
Assay	Hemagglutinin subtype	Virus isolate	1	2	3	4	5	6	7	8	10	11	13	15
IPMA	H1	A/duck/Germersheim/R30/06 (H1N1)	-	-	-	-	-	-	-	-	-	-	-	-
ST-606 cells infected with viruses	H2	A/guinea fowl/Ger/DZ3/85 (H2N2)	-	-	-	-	-	-	-	-	+	+	-	-
	H5	A/Vietnam/1194/2004 (NiBrg14) (H5N1, HP)	+	+	+	+	+	-	+	+	+	+	-	-
		A/teal/Föhr/Wv632/05 (H5N1, LP)	+	+	+	+	+	+	+	+	+	+	+	+
		A/goose/Manitoba/428/06 (H5N2)	+	+	+	+	+	+	+	+	+	+	+	+
		A/duck/Potsdam/1402/86 (H5N2)	+	+	+	+	+	-	+	+	+	+	+	-
		A/Mallard/Ger/Wv476/04 (H5N2)	+	+	+	+	+	+	+	+	+	+	+	+
		A/Mallard/Ger/Wv1349/03 (H5N3)	+	+	+	+	+	+	+	+	+	+	+	+
		A/duck/Potsdam/2216/84 (H5N6)	+	+	+	+	+	+	+	+	+	+	+	+
		A/chicken/Italy/22/98 (H5N9)	+	+	+	+	+	+	+	+	+	+	+	+
		A/mallard/B.C./544/05 (H5N9)	+	+	+	+	+	+	+	+	+	+	+	+
	H6	A/turkey/Germany/617/07 (H6N2)	+	-	-	-	+	-	-	-	-	-	-	-
High-Five cells expressing recombinant target	H5	A/chicken/Egypt/0879-NLQP/2008 R737/09	-	-	-	-	-	+	-	+	-	-	-	-
	H5	A/Canada goose/Germany/71/06	+	+	+	+	+	+	-	+	+	+	+	+

A total of eleven different hemagglutinins of the H5 subtypes were analysed and the vast majority of the antibodies bound well to these antigens. Only minor heterosubtypic binding to H2 (JM7_10 and JM7_11) and H6 (JM7_1 and JM7_5) was observed in this assay. As clones JM7_5 and JM7_3 are identical in amino acid sequence no clear result for this antibody clone can be stated with regard to its H6 binding. None of the antibodies bound to H1. All antibodies detected a broad spectrum of H5 expressed on infected or recombinantly H5 expressing cells but only two antibody clones, JM7_8 and JM7_10 detected all eleven H5 viruses including the highly mutated Egyptian escape mutant strain (A/chicken/Egypt/0879-NLQP/2008 R737/09).

The antibody clone JM7_8 was the only antibody that bound to all eleven different H5 without any cross-reactivity with other hemagglutinin subtypes. Binding of JM7_8 antibody to recombinant insect cells expressing H5 variants of highly pathogenic avian influenza viruses is shown in Fig 30 and to a selection of H5 variants of low pathogenic avian influenza viruses in Fig 31. Compared to an chicken anti-A/chicken/Egypt/0879-NLQP/2008-R737/09 serum antibody JM7_8 shows comparable binding to cells presenting H5 derived from A/chicken/Egypt/0879-NLQP/2008-R737/09 on their surface.

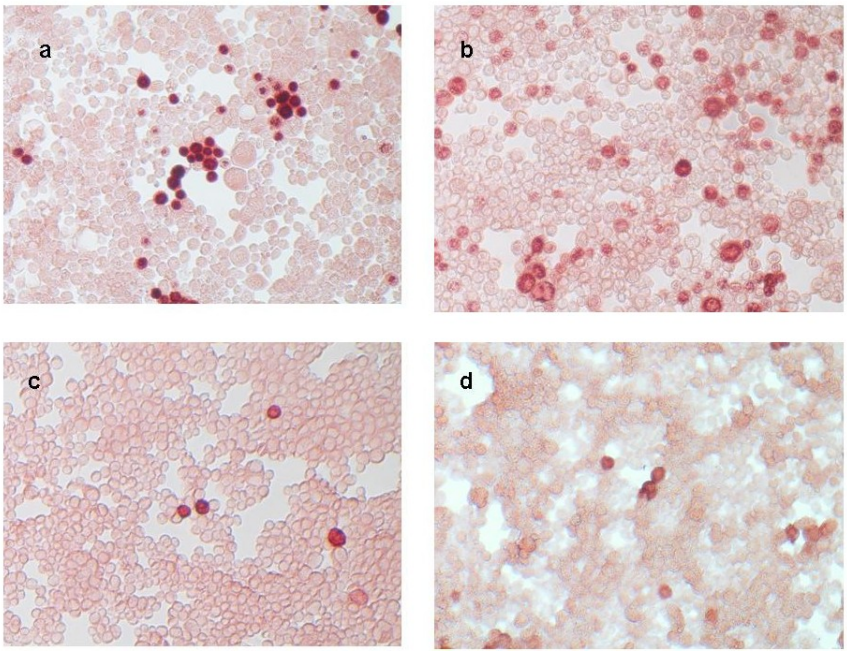


Figure 30: IPMA of clone JM7_8 scFv-hIgG1Fc on High Five insect cells expressing H5 of different highly pathogenic avian influenza strains.
ScFv-hIgG1Fc antibodies on High Five insect cells recombinant expressingH5 of a) A/chicken/Egypt/0879-NLQP/2008-R737/09 (H5N1 HP) b) A/Canada goose/Germany/71/06 H5N1 HP c) A/Canada goose/Germany/71/06 (H5N1)detected by mAb 8292 (positive control), d) A/chicken/Egypt/0879-NLQP/2008-R737/09 (H5N1 HP) detected by anti R737 chicken serum

(positive control); secondary antibody for a) and b): anti-humanIgG Fc spezific HRP antibody; secondary anribody for c) and d): anti-chicken Fc specific HRP antibody

An additional positive control for these stainings was a monoclonal antibody directed against the internal virus nucleoprotein NP (Fig 30c). Staining is comparable for antibody JM7_8 and anti-NP antibody on cells presenting the same H5 (Fig30b,c).)

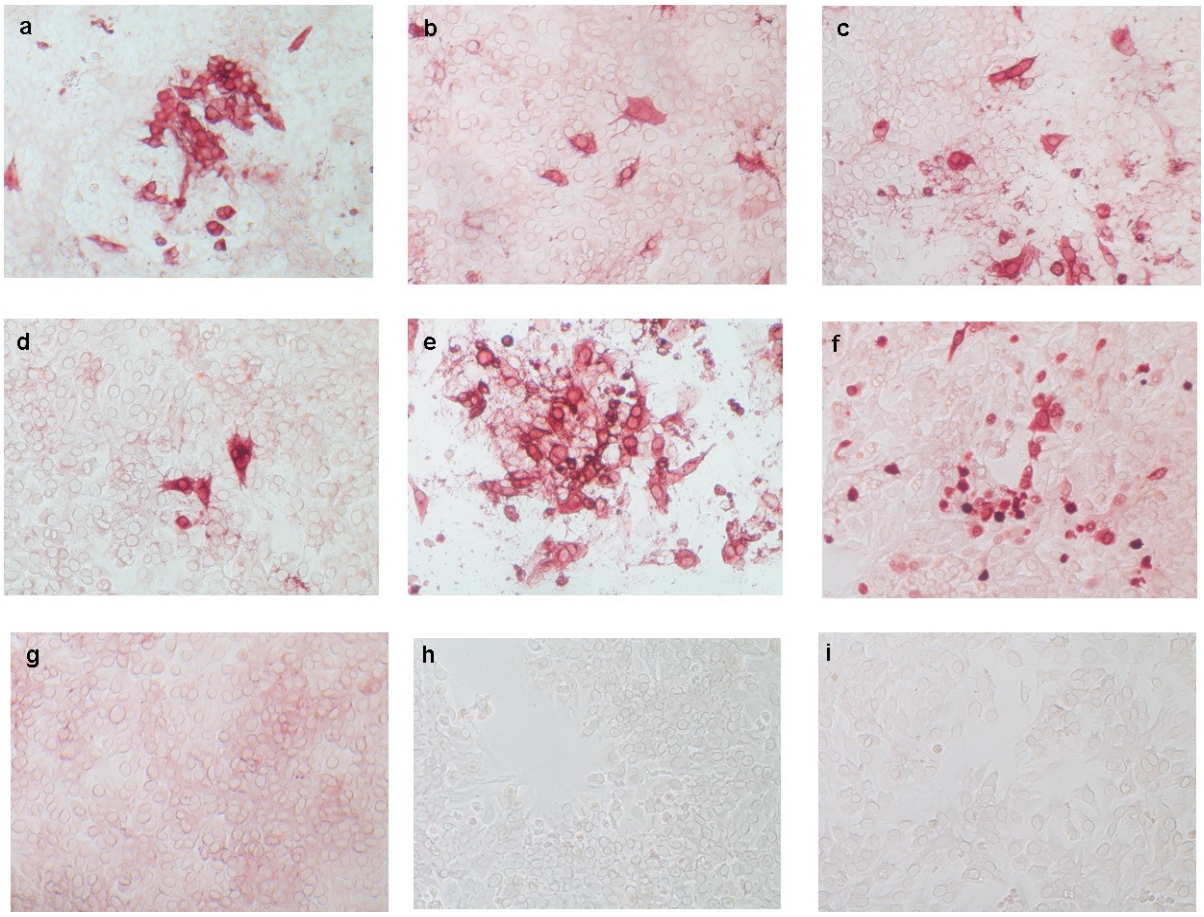


Figure 31: IPMA of clone JM7_8 scFv-hIgG1Fc on swine testis cells infected with different low pathogenic avian influenza strains.
ScFv-hIgG1Fc antibodies on recombinant swine testis cells infected with a) A/teal/Föhr/Wv632/05 (H5N1), b) A/chicken/Italy/22/98 (H5N9), c) A/duck/Potsdam/2216/84 (H5N6), d) A/goose/Manitoba/428/06 (H5N2), e) A/mallard/B.C./544/05 (H5N9), f)positive control A/turkey/Germany/617/07 (H6N2) anti-NP antibody, negative controls: g) A/turkey/Germany/617/07 (H6N2),h) uninfected swine testis cells, i) A/chicken/Italy/22/98 (H5N9) anti-humanIgG Fc spezific HRP antibody

Staining of H5 of infected swine testis cells is more directed to the surface of the cells (Fig 31a, b, c, e, and Fig 32a, b, c, d) compared to the anti-NP antibody used for positive control (Fig 31f) where staining is more prominent in the inner parts of the cell, which is consistent with the predominant localization of the respective target proteins.

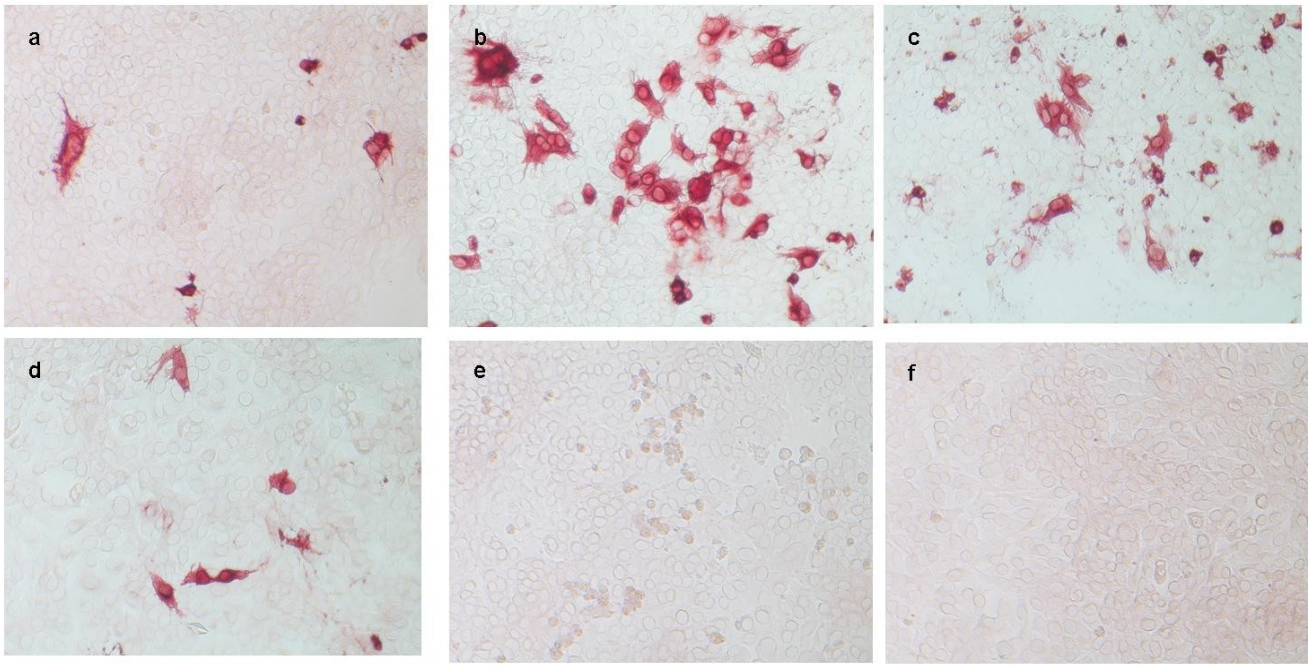


Figure 32: IPMA of clone JM7_1 scFv-hIgG1Fc on swine testis cells infected with different low pathogenic avian influenza strains.
ScFv-hIgG1Fc antibodies on recombinant swine testis cells infected with a) A/Mallard/Ger/Wv1349/03 (H5N3) b) A/teal/Föhr/Wv632/05 (H5N1, LP) c) A/duck/Potsdam/1402/86 (H5N2) d) A/goose/Manitoba/428/06 (H5N2) e) A/duck/Germersheim/R30/06 (H1N1) f) uninfected swine testis cells with anti-humanIgG Fc spezific HRP antibody

JM7_1-hIgG1Fc shows a broad H5 detection spectrum of all low pathogenic H5 variants but lacks detection of the highly mutated Egyptian escape mutant A/chicken/Egypt/0879-NLQP/2008 R737/09and has minor cross-reactivity with H6. Binding of JM7_1-hIgG1Fc to a selection of H5 from low pathogenic influenza viruses is shown in Fig 32. Negative controls are shown in Fig 31 g-i with a non-H5 virus (H6N2) used for infection (Fig 31g), with the anti-humanFc-HRP conjugate alone on infected cells(Fig 31i). Uninfected swine testis cells stained with JM7_1-hIgG1Fc and anti-humanFc-HRP conjugate, remained completely unstained(Fig 31h).

6.7 Electron microscopy TEM and SEM

Influenza virus strain A/teal/Föhr/Wv63/05 was propagated in chicken eggs and specific binding of isolated scFv-hIgG1Fc antibodies was demonstrated for some antibodies in transmission electron microscopy (TEM). ScFv-Fc antibodies were labeled with 15 nm protein-A-gold particles. Specific binding of selected antibodies (black dots) JM7_3 and JM7_8 to A/teal/Föhr/Wv63/05 virus surface proteins was demonstrated for low (25 ng/mL; Fig 33e, f) and higher (25 µg/mL; Fig 33g-k) antibody concentration. Resolution is higher for Fig 33a-c and Fig 33g-k than for Fig 33d-f.

Sample preparation and antibody labelling were performed in cooperation with Prof. Dr. Manfred Rohde (HZI), pictures were taken by Manfred Rohde and kindly provided. Specific binding of isolated chicken antibodies to whole influenza viruses is strongly indicated by these results.

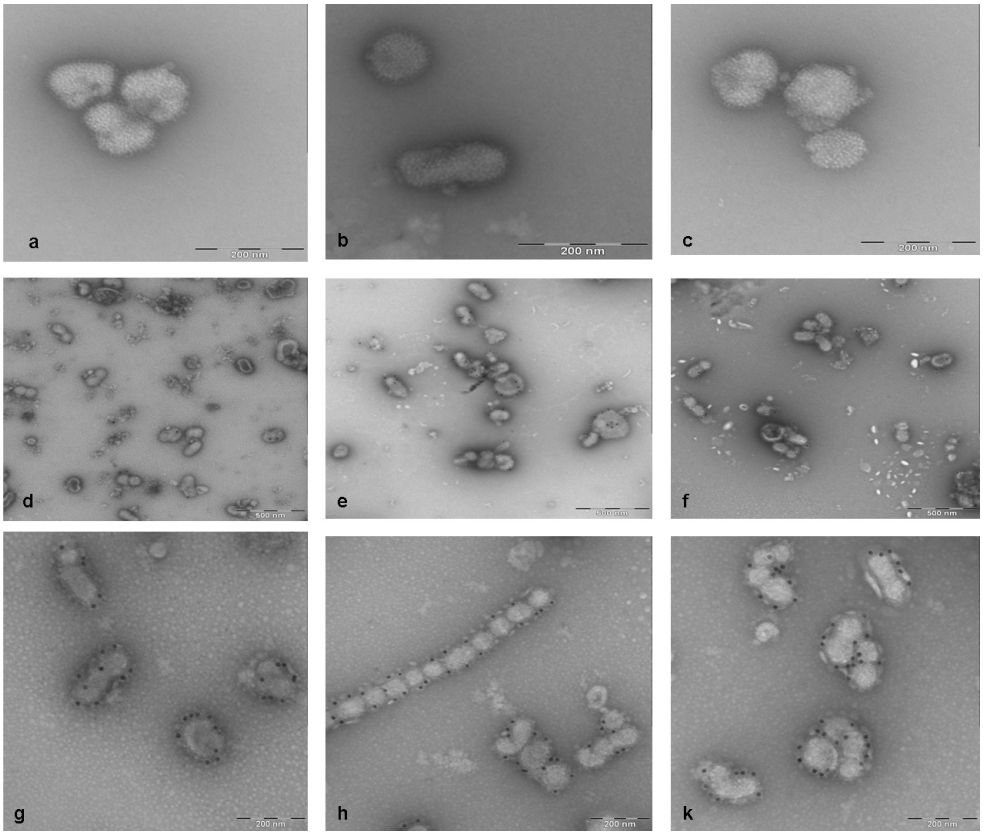


Figure 33: Transmission electron microscopy of influenza virus A/teal/Föhr/Wv632/05 and scFv-hIgG1Fc antibodies bound to the virus.
A/teal/Föhr/Wv632/05 produced in embryonated chicken eggs after purification by ultracentrifugation (a-c), background binding of 15 nm protein-A-gold particles on virus preparation (d), – e) JM7_3 (25ng), f) JM7_8 (25ng), g-k) JM7_3 (250µg)

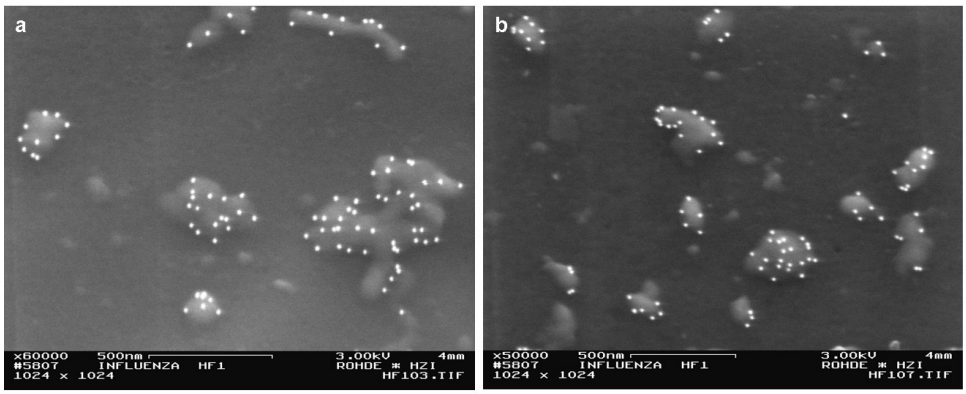


Figure 34: Scanning electron microscopy of JM7_3 scFv-hIgG1Fc antibodybound to influenza virusA/teal/Föhr/Wv632/05.
A/teal/Föhr/Wv632/05 produced in embryonated chicken eggs after purification by ultracentrifugation detected with 15 nm protein-A-gold labeled JM7_3 scFv-hIgG1Fc antibody (a and b)

TEM results were confirmed by scanning electron microscopy (SEM) showing gold-

labeled antibodies (white dots) binding to the surface of influenza virions (Fig 34). Binding of several identical antibodies to the surface of a single virion was shown in TEM and SEM (Figs 33, 34).

6.7.1 Hemagglutination Inhibition (HI)

Since all identified antibodies bound to the head-like HA1 part of the hemagglutinin and this part is also target of most of the neutralizing antibodies hemagglutination inhibition tests were performed to analyse identified antibodies regarding their HI activity. The HI test probes the ability of the isolated antibodies to block agglutinating effects of viral hemagglutinin on erythrocytes. Typically, a positive HI test is a first hint on the virus neutralizing potential of an antibody.

These tests were performed by Cornelia Illing in the group of Prof. Dr. Timm Harder.

None of the identified H5 binders displayed HI activity against any of the H5-influenza viruses used in this assay (Tab 14).

Table 14: Hemagglutination Inhibition Assay (HI) for anti-H5 scFv-hIgG1Fc antibodies.
HI was performed using freshly prepared chicken blood and a serial dilution of indicated virus strains and of H5 specific scFv-hIgG1Fc antibodies. No hemagglutination inhibition is indicated by a '-',

Virus	reactivity of H5 specific scFv-hFc														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A/Teal/England/7394-2806/06 (H5N3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Swan/Germany/R65/06 (H5N1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Vietnam/1197/2004 (NiBrg14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/teal/Föhr/Wv632/05 (H5N1, LP)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

6.7.2 Virus Neutralization Assay (VN)

To further analyse neutralization activity of identified chicken antibodies microneutralization assays were performed in a 96 well scale using $MDCK^+$ cells. Viral infectious dosis was 100 $TCID_{50}$. Measurements were performed in quadruplets.

According to the standard procedure applied at the FLI (first adding trypsin to the seeded cells and second preincubated antibody-virus-mixture) only antibody JM7-3 showed broad neutralizing activity for eight different H5-influenza strains. Except for A/mallard/B.C/5445/05 (H5N9) all H5 viruses assayed were neutralized by this antibody. Neutralizing activity was restrained to viruses of H5 subtype. Viruses of H1, H2 and H6 subtypes were not neutralized.

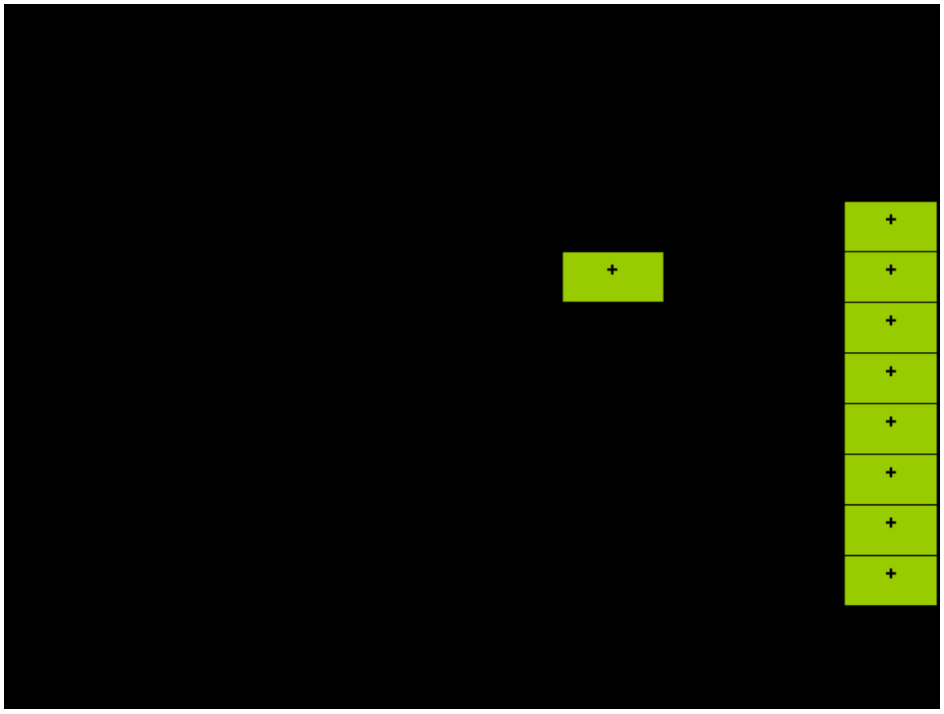
The standard procedure was altered and trypsin was added after a six hours incubation of preincubated antibody-virus-mixture and seeded cells. Under these conditions additionally

JM7_8 could be identified as a neutralizing antibody (Tab. 15). Neutralization was assayed for JM7_8 only using virus A/teal/Föhr/Wv632/05 and A/mallard/B.C./544/05.

Antibody titration was performed for both antibodies JM7_8 and JM7_3 to a minimum of 0.13 nM which was the end point of titration. Both antibodies showed still complete neutralization at this low antibody concentration.

Additional analyses revealed a complete digest of scFv-mIgG2cFc antibodies JM7-8, JM7_1 and JM7_10 and an only partial but significant degradation of JM7_3 after a 1 h incubation with trypsin at 37 °C (data not shown).

Table 15: Virus microneutralization assay for isolated anti-H5 scFv-mIgG2cFc antibodies.
The assay was performed using MDCK$^+$ cells and indicated viruses were used at 100 $TCID_{50}$, '+'complete neutralization of all four wells, '-' no neutralization, empty fields: assay not performed. 100 $TCID_{50}$ was confirmed by titration for each virus in the same assay.



6.8 Epitope mapping for two H5 specific antibodies

For antibodies JM7_8 and JM7_1 epitope mapping was successfully performedusing peptide spot membrane. Membrane was designed on the basis of the H5 sequence of A/Anhui/1/2005. Consistent with the results from immunoblot using virus preparations (Fig 24) both epitopes are located in the HA1 part of the hemagglutinin molecule.

The epitope of JM7_8 revealed to be a six amino acid strech with the amino acid sequence FPNVVW at hemagglutinin positions 146-152 (PDB accession number 2FK0). The epitope appears to be rather hidden in the crystal structure (Fig 35.) Location of the epitope in top and side view of the crystal structure is shown as well as detection spots on

the peptide spot membrane and derived amino acid sequence of the respective epitope.

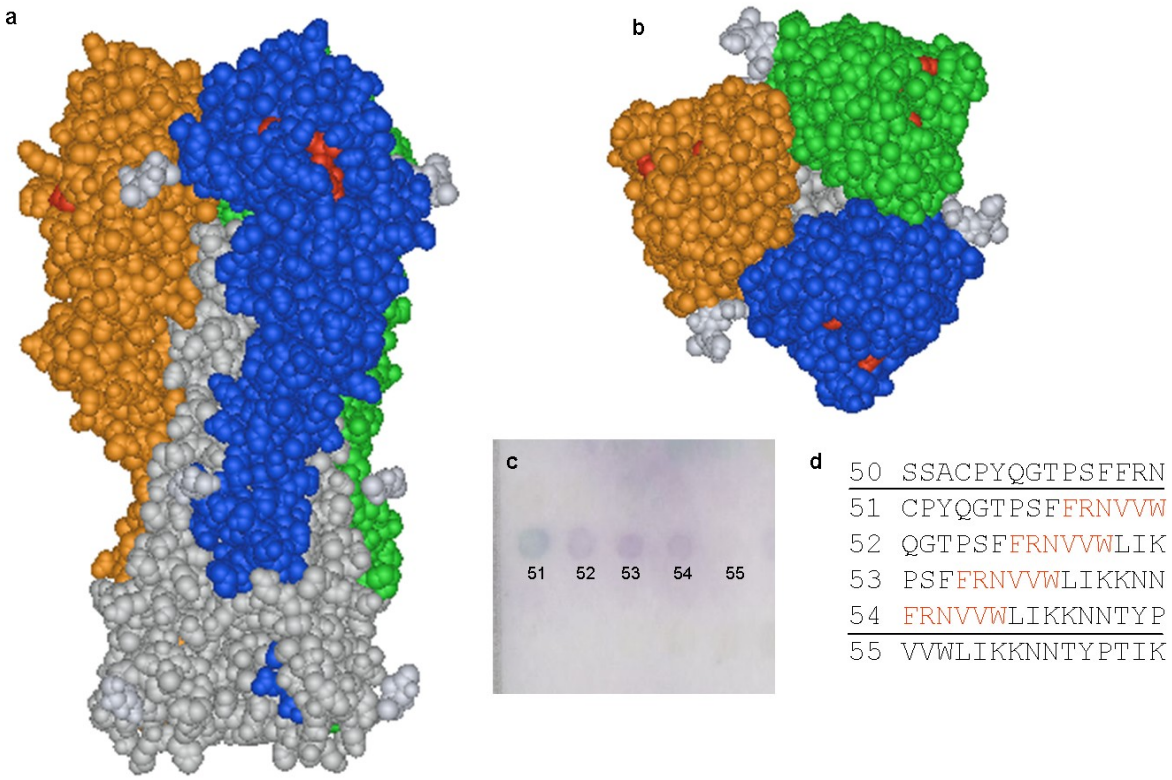


Figure 35: Three dimensional picture of the HA trimers of avian hemagglutinin and epitope of antibody clone JM7_8.
A) and B) represent thwo different views (A – side view; B – top view) of HA trimers; HA1 molecules are highlighted (orange, green, blue); epitope of JM7_8 antibody is shown in red. Epitope membrane is shown in C) and amino acid sequence of peptides detected by the antibody is shown in D). Assumed antibody peptide is shown in red. (molecular structure reference: 2IBX pdb VN1194 Yamanada)

For antibody clone clone JM7_1 two different sites of antibody binding were detected on the peptide spot membrane Fig 36. One epitope including peptides 74 – 76 with the amino acid sequence VPKIATRSK the other epitope comprises peptide numbers 111 – 115 with the amino acid sequence ERRRKR, which includes the cleavage site of the hemagglutinin. But as shown in IPMA and immunoblot assays JM7_1 binds equally well to H5 from highly and low pathogenic influenza viruses which show major differences in their cleavage sites. This antibody cannot provide specific binding of the influenza cleavage site region.

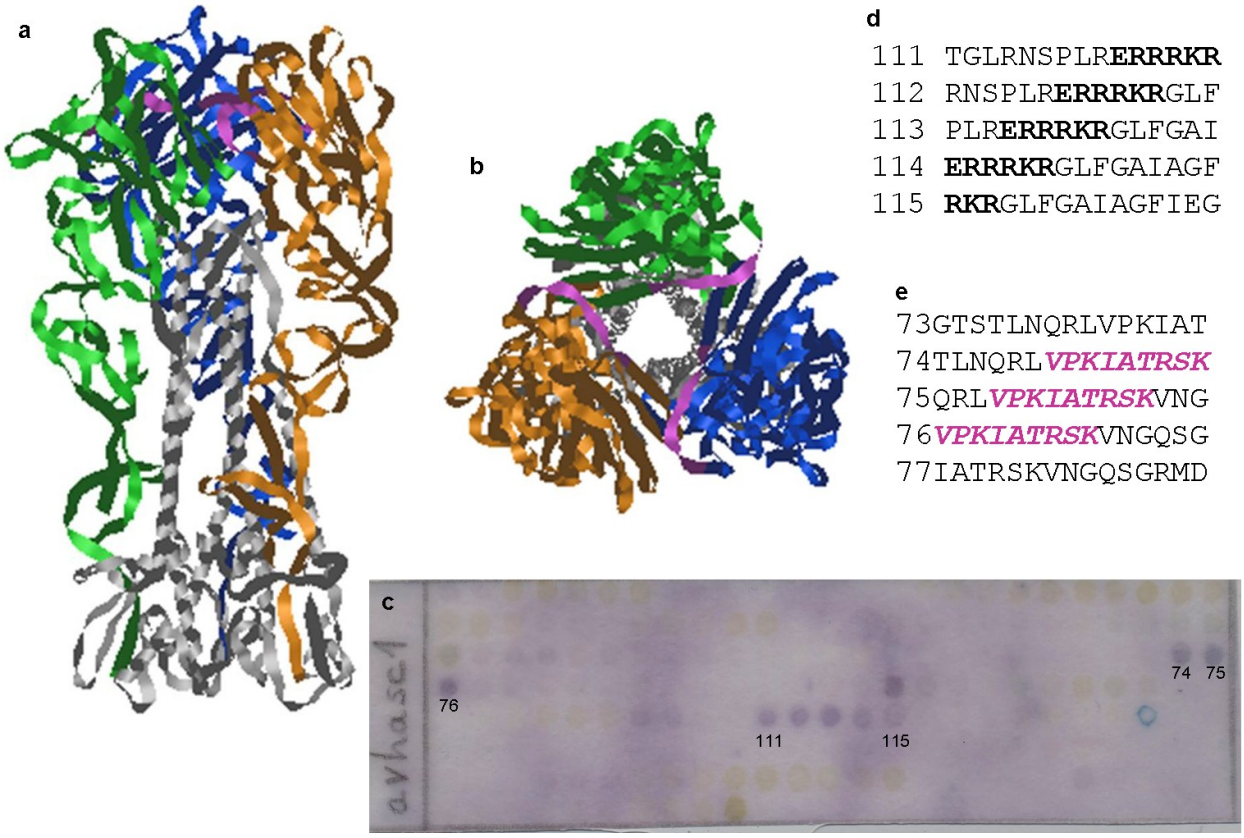


Figure 36: Three dimensional picture of the HA trimers of avian hemagglutinin and epitope of antibody JM7_1.
a) and b) represent thwo different views (A – side view; B – top view) of HA trimers; HA1 molecules are highlighted (orange, green, blue); epitope of JM7_1 antibody is shown in magenta. epitope membrane is shown in c) and Amino acid sequence of peptides detected by the antibody is shown in d for peptides 111-115 and in e) for peptides 74-76. Assumed antibody peptide is shown in magenta. molecular structure reference: 2IBX pdb VN1194 [190]

7 Discussion

Highly pathogenic H5N1 avian influenza virus (HPAIV H5N1) devastates the poultry industry worldwide causing enormous economic losses. In addition, the virus poses a significant pandemic threat to human health. Due to the vital role of hemagglutinin protein (HA) in viral infections and in escape from treatment and control, different strategies targeting the HA have been developed for diagnosis, vaccine design and therapeutic applications. Recombinant neutralizing antibodies (rnAbs) specific to the HA protein were used for diagnostic purposes and protection studies. A rapid subtype specific detection system compatible with field application for highly pathogenic avian influenza viruses will be useful in surveillance of the rapidly changing viruses but depends on highly conserved epitopes allowing the discrimination of certain virus subtypes, especially H5 and H7, the only highly pathogenic avian influenza virus suptypes observed to date in the field.

This work aimed at the isolation and characterization of broadly reactive antibodies specific for influenza viruses of the H5 subtype.

From the universal human naïve antibody phage libraries HAL7 and HAL 8 [177] no H5 specific antibodies were isolated, although about 300 antibody clones were analysed after three different panning campaigns. Obviously, the isolation of anti-H5 antibodies from human naïve libraries poses a challenge. Published data on human naïve antibody libraries for the selection of anti-H5 antibodies are rare. The human antibody libraries of Throsby *et al.* and Sun *et al.* were generated from vaccinated or naturally infected donors, respectively, and therefore are no naïve human libraries[191,192]. Lim and coworkers [193] have used the commercial human Fab library from Humanyx (Singapore) of undeclaredimmune status of donors [§]. Of similar undisclosed immune origin has been a human scFv antibody library of Yang *et al*[194]. Although a successful isolation of neutralizing anti-H5 antibodies from all these human antibody libraries has been shown, these libraries may not be comparable to human naïve antibody phage libraries like HAL7 and HAL 4.

Libraries generated from H5 infected or vaccinated humans [191,192] evidently have a

[§]Lim *et al.*declared the use of a **naïve**human Fab antibody library HX01 from Humanyx (Singapore). This library specification cannot be deduced from information provided on the homepage of the company.

non-naïve antibody gene repertoire. Likewise, one may assume a similar post contact status with regard to H5 for undisclosed donors from which antibody libraries in Asian institutes were constructed[193,194]. In Asia the increased contact to H5N1 viruses due to frequent H5N1 outbreaks in poultry and living of humans in close proximity to poultry clearly differ from the situation in Europe. While antibody genes of European donors predominate the antibody libraries HAL7 and HAL4, a comparable bias towards antibody genes of Asian origin may be supposed for the libraries of Yang *et al.* and Lim *et al*. These aspects may have facilitated the isolation of anti-H5 antibodies from these libraries. Immune antibody libraries are a well accepted tool to increase the probability of success in antibody isolation to a given antigen[195].

7.1 High quality anti-H5 immune antibody phage libraries were obtained from chickens

As birds are the natural host for H5 influenza viruses and are susceptible, chickens were chosen to be immunized and boosted with different H5 influenza viruses. In addition, due to conserved regions flanking chicken antibody genes chicken antibody libraries are easily to be constructed. Only two sets of oligonucleotides were sufficient to amplify chicken antibody gene fragments from RNA[185]. Two independent libraries were constructed from peripheral blood lymphocytes and from splenic RNA to access the complete diversitiy of the antibody repertoire of the immunized donors as reported before [185,196–200]. Random pairing of V_H and V_L gene fragments was allowed thus creating an artificial antibody diversity without assured conservation of the original donor V_H-V_L combinations. Unexpectedly, degradation of antibody gene fragments occurred due to restriction sites within the chicken antibody genes. Deleterious restriction sites were cleaved by the enzymes NcoI and MluI immanent to the employed phage display vector. This drawback has not been described in literature, as others use different restriction enzymes like ApaLI/NotI[196], SfiI/NotI[200], Sapats SalI/NotI and AscI/XbaI[198], NotI[201] and SfiI[197,202] for chicken antibody library construction. Hence, a reduction of antibody gene diversity for the chicken libraries generated in this work must be taken into consideration. Nevertheless, the quality of the anti-H5 chicken immune libraries was underlined by a total number of up to 10^7 independent clones after transformation of antibody gene containing phagemid vectors, up to 100 % correct fragment size and a clear presentation of scFv antibodies on the surface of the phage along with a high phage titre of both antibody

phage libraries. Although the human naïve antibody libraries HAL7 and HAL4 show a larger complexicity (10^9 individual clones) than the constructed chicken anti-H5 immune libraries, implementation of immune libraries was more successful in this project. Numerous anti-H5 antibodies have already been isolated from human antibody libraries [191,192,203,204]. Yet, as most of them either exhibited cross-reactivity with other hemagglutinin subtypes or did not broadly detect H5, it is likely that they would not have fullfilled the aim of the project.

To direct antibody binding characteristics towards the desired broad H5-binding activity four different H5 antigens were introduced into the selection. Two phylogenetic distant avian influenza viruses influenced the chicken in vivo selection after immunization and two different highly pathogenic recombinant H5 antigens were used in the in vitro selection process. The latter two different H5 antigens, originating from viruses of human and avian origin, have been implemented in two separate panning campaigns. Two rounds of pannings were sufficient for both targets to isolate a total of twelve different antibody clones binding both H5 variants specifically. This is in contrast to the selection strategies based on a single H5 antigen [191,192,194,202]. Similar to the selection strategy using heterologous H5 antigens presented in this work, Lim *et al.* have used two different recombinant H5 antigens[193]. Of note, the majority of their isolated antibodies bound to linear epitopes. In contrast to this, the dominance of conformational epitopes of selected antibodies using different virus-like particles is demonstrated in the same work. *Wang et al*. have shown that broadly protective antibodys against H3 were elicited after sequential immunization with four different viruses[205]. Therefore, the applied extented heterologous selection strategy herein may have contributed to both – the isolation of antibodies with broad H5 specificity on one hand and the isolation of antibodies exclusively binding linear epitopes on the other hand.

7.2 Isolated antibodies specifically bind to linear epitopes and detect H5 influenza viruses wihout or with low cross-reactivity towards different HA subtypes

The two different H5 antigens applied in selection and screening were derived from human (A/Anhui/1/2005) or avian (A/Canadagoose/Germany/71/06) influenza viruses, respectively. The human antigen was monomeric whereas the avian H5 was a trimer that was captured from baculoviral production supernantant using streptavidin beads. Although only bead immobilized human H5 was used in the

selection process the vast majority of selected antibodies bound to plate immobilized human H5 as well as to bead immobilized human H5. Hence it can be either concluded that presentation of H5 on beads and plate did not differ very much in the performed assays under the applied conditions or that, if presentation differs, bound epitopes are accessible in both ways of presentation. The importance of immobilization strategies for the isolation of antibodies binding certain conformational epitopes has been described earlier, demonstrating that bead immobilized recombinant antigens may provide a more naturally shaped antigen surface[206] . A total of twelve antibodies bound to both recombinant H5 antigens in ELISA. All twelve antibodies detected human recombinant H5 antigen and the majority of them also avian recombinant H5 antigen as well as two further H5 viruses propagated in chicken eggs in immunoblots under denaturing conditions. From these results it can be concluded that all selected antibodies bind to linear epitopes since conformational epitopes tend to be destroyed under these conditions. In addition all epitopes are located in the head like part of the hemagglutinin, since exclusively HA0 and HA1 were detected in immunoblots. Unexpectedly, two antibodies (JM7_6 and JM7_8) detected the hemagglutinin of a highly pathogenic H5 escape mutant (A/chicken/Egypt/0879-NLQP/2008-R737/09), that has amassed numerous mutations in its hemagglutinin[120]. Thus, at least for these two antibodies broad H5 detection and highly conserved epitopes can be assumed.Highly conserved subtype specific epitopes in the highly variable head-like part of the hemagglutinin are rare. In contrast to this, strain specific neutralization of influenza viruses is based to a large extent on antibodies binding near receptor binding sites[207,208]. The well accessible HA1 part plays a major role in antigenic drift and frequent mutations in this part of the protein assist the virus in escaping from immune responses[8,50]. Nevertheless, few highly conserved epitopes had been mapped to the HA1 part[207,209]. In contrast, broadly neutralizing hemagglutinin specific antibodies are more likely to bind to the more conserved stalk-like structure of the hemagglutinin[210–213].

Furthermore, specificity of isolated antibodies was demonstrated using nine different low pathogenic avian H5 influenza viruses and three H5 antigens of highly pathogenic viruses by specific staining of infected or recombinant H5 expressing cells (IPMA), respectively. Eight individual antibodies detected all low pathogenic H5 antigens. The highly pathogenic avian H5 antigen, already used in selections was detected by all of them in this assay. Minor cross-reactivity was observed for a total

of four antibodies with subtypes H2 or H6. None of the clones bound to H1 in the virus infection assay or H7 in immunoblot. Low background staining observed in this assay underlines the specificity of the isolated antibodies. Specific antibody binding to the surface of influenza viruses was clearly shown in electron microscopic pictures obtained with purified H5 influenza virus preparations and immunogold labeled antibodies. Once more, low antibody background binding was demonstrated based on both, a good virus preparation with low amount of free hemagglutinin and virus debris and highly specific anti-H5 antibodies.

Furthermore, as a proof of principle for an antibody based detection system capture ELISA with scFv-Fc antibodies differing at least in their Fc-part were carried out. Eight out of twelve different H5 influenza viruses or respective antigens could be detected. No cross-reactivity with H1 and H2 viruses has been observed which was consistent with results obtained from cell infection assay. Under mild denaturing conditions an improved detection of the virus A/mallard/B.C/544/05 H5N9 could be obtained whereas other viruses remained undetectable under these conditions. Moreover, for A/teal/Föhr/Wv632/05 these conditions turned out do be unfavourable since detection of this virus was abolished then. One may speculate that partial unfolding of the hemagglutinin could have caused these effects. Accessibility of antibody epitopes may be different and dependent on structural integrity of the hemagglutinin at least to a certain extent. To obtain a complete virus detection spectrum detection should be performed under both conditions, mild denaturing and non-denaturing. Surprisingly, not all antibody combinations were capable of detecting viruses from propagated chicken egg samples. Influenza viruses present several hundred hemagglutinin molecule entities on their surface. Hence, all antibody combinations should work as capture and detection antibodies in ELISA. As this could not be demonstrated for all antibodies, one may conclude that free soluble hemagglutinin possessing each antibody binding site only once per molecule is detected from the virus samplesrather than complete viruses. Only in the case that the detecting antibody epitope is still accessible while the capturing antibody has already bound to the soluble antigen, capture ELISA will work. Few antigen capture ELISA (AC-ELISA) using chicken egg derived virus preparations have been described. He et al. demonstrated the use of two different antibody formats (IgG and IgM) binding different epitopes for capturing and pooled hyperimmune sera for detection of H5 viruses[214]. The applied antibody amount was comparable to the work

presented here with 100 ng capture and 800 ng detection antibody. In this AC-ELISA four H5 viruses were discriminated from five viruses of different HA subtypes and the detection limit was detemined to be 124 $TCID_{50}$. A similar AC-ELISA was developed for the detection of H7 viruses with a detection limit of 10^2-10^3 $TCID_{50}$[86]. In the case that soluble hemagglutinin interferes with capturing of viruses the determination of a detection limit with a $TCID_{50}$ or a hemagglutination titre may not aid. Since both values estimated whole virus particles which in turn appear not to be detected in the AC-ELISA described in this work, recombinant human H5 antigen was used in AC-ELISA. Detection limit for this antigen was below 75 ng per well. Under the assumption that the same epitope theoretically exists only once per molecule antibody pairs sharing the same scFv fragment should not be capable of detecting soluble recombinant H5 antigen. Unexpectedly, those homologous antibody pairs were effective in detection of the used recombinant antigen. Hence, a spontaneous multimerization of the soluble recombinant antigen must be assumedSpontaneous multimerization of soluble recombinant hemagglutinin has been observed (J. Skehel, personal comm.). The estimated detection limit cannot be compared with published data. The described challenge of interfering soluble hemagglutinin is circumvented by an AC-ELISA using an anti-H5 antibody for capturing and an anti-N1 antibody for detection[215]. As both antibodies detect highly conserved epitopes, nevertheless, this assay is restricted to H5N1 viruses and exclusively detects virus particles or membrane fragments harbouring both HA and NA molecules. In contrast, the major advantage of a detection system based on soluble hemagglutinin and linear epitopes is its increased robustness in field application. Reduction of viral integrity and partial unfolding of viral hemagglutinin in natural samples may frequently occur.

A membrane based detection format was assayed as an additional proof of principle. A total of 100 ng membrane bound antibody was sufficient to give a positive signalafter detection with a respective antibody possessing a different Fc-part. Hong et al. have demonstrated a detection system combining the nitrocellulose membrane immobilized capture antibody with a magnetic bead immobilized detection antibody[216]. This biosensor allows the detection of viral protein in the picogram range via magnetic sensors.

Beyond antibody specificity and detection limit, antibody affinity is a further important parameter for the design of detection systems. EC_{50} values of all analysed antibodies reached subnanomolar ranges. Due to restriction in antigen availability and instability

of the chicken scFv antibodies EC_{50} values were calculated from titration ELISA with scFv-hIgG1Fc antibodies and represent apparent affinities. Published antibody affinities based on biosensor studies and H1 hemagglutinin range from 1.7 x 10^{-7} – 4.8 x 10^{-11} M [207,217] and values within this range (5 x 10^{-10} M and 1.7 x 10^{-10} M) were obtained for neutralizing antibodies binding the receptor-binding site of H3[218]. These biosensor based values cannot be directly compared to obtained EC_{50} values from titration ELISA. However, the best EC_{50} value of 1.4 x 10^{-10} M for antibody clone JM7_1 indicate very good affinities of the isolated anti-H5 antibodies. High affinities are indispensible in detection systems when minor or even trace amounts of virus or antigens thereof shall be detected.

7.3 Two antibodies specifically neutralize H5 influenza viruses

Virus neutralization and thus protective potential of antibodies with broadest H5 binding spectrum was analysed in HI and virus neutralization tests. Two of the four tested antibodies showed virus neutralization restricted to H5 viruses. No 50 % endpoint tiration titres were determined since both antibodies JM7_8 and JM7_3 still showed complete neutralization at 1.3 x 10^{-10} M and 100 $TCID_{50}$. Antibody neutralization titre must therefore be below this value. Throsby *et al*. have published IC50 values of 3 x 10^{-9} for neutralizing antibodies with regard to H5 viruses and 1.96 x 10^{-7} for heterosubtypic viruses[192]. Antibody clone JM7_3 neutralization was successful with standard procedure for eight of the analysed nine viruses. Only A/mallard/B.C./544/09 H5N9 virus was not neutralized. Control viruses of the hemagglutinin subtypes H1, H2 and H6 were not neutralized. For clone JM7_8 no neutralization was observed with standard procedure. Following the assumption that trypsin facilitating the infection of MDCK cells in the case of low pathogenic influenza viruses may degrade antibodies and thus prevent neutralization, virus neutralization procedure was altered. Susceptibility of IgG to proteolytic digest with trypsin has been described earlier by Brown *et al.*[219]. Surprisingly, no published comment on the observed antibody degradation during virus neutralisation assay could be found. After a six hours delay in trypsin addition antibody JM7_8 conferred neutralization at least for A/teal/Föhr/Wv632/05 to the same low antibody neutralization titre as JM7_3 at the endpoint of titration. Complete degradation of applied antibodies due to the proteolytic activity of trypsin was shown in separate assays with antibody clone JM7_3 showing partial resistance to tryptic digest. This could explain why standard neutralization procedure was successful for this antibody.

From the neutralizing effect of the antibodies and the elucidated HA1 specificity of both of them can be concluded that the epitopes of JM7_8 and JM7_3 interfere with the receptor binding site of the hemagglutinin. However, all antibodies were negative in HI test. This result is contradictory to the virus neutralization test results since both assays analyse the inhibition of the receptor binding activity of viral hemagglutinin. This failure in HI may be either due to the structure and potentially reduced flexibility of the artificial chimeric antibodies (chicken scFv and human Fc) or due to undisclosed factors contributing to a less effective interplay of chicken erythrocytes with human serum in HI reported earlier [220]. Moreover, goose erythrocytes were reported to be beneficial in hemagglutination assay with H5N1 viruses[221]. The failure of HI assay could therefore be false negative results caused by assay immanent factors. The observation that human H3 influenza viruses have lost the ability to agglutinate chicken erythrocytes is of further interest[222]. This indicates that the hemagglutination and hemagglutination inhibition assay applied to influenza viruses may need additional revision with regard to reliability.

7.4 Antibody sequences

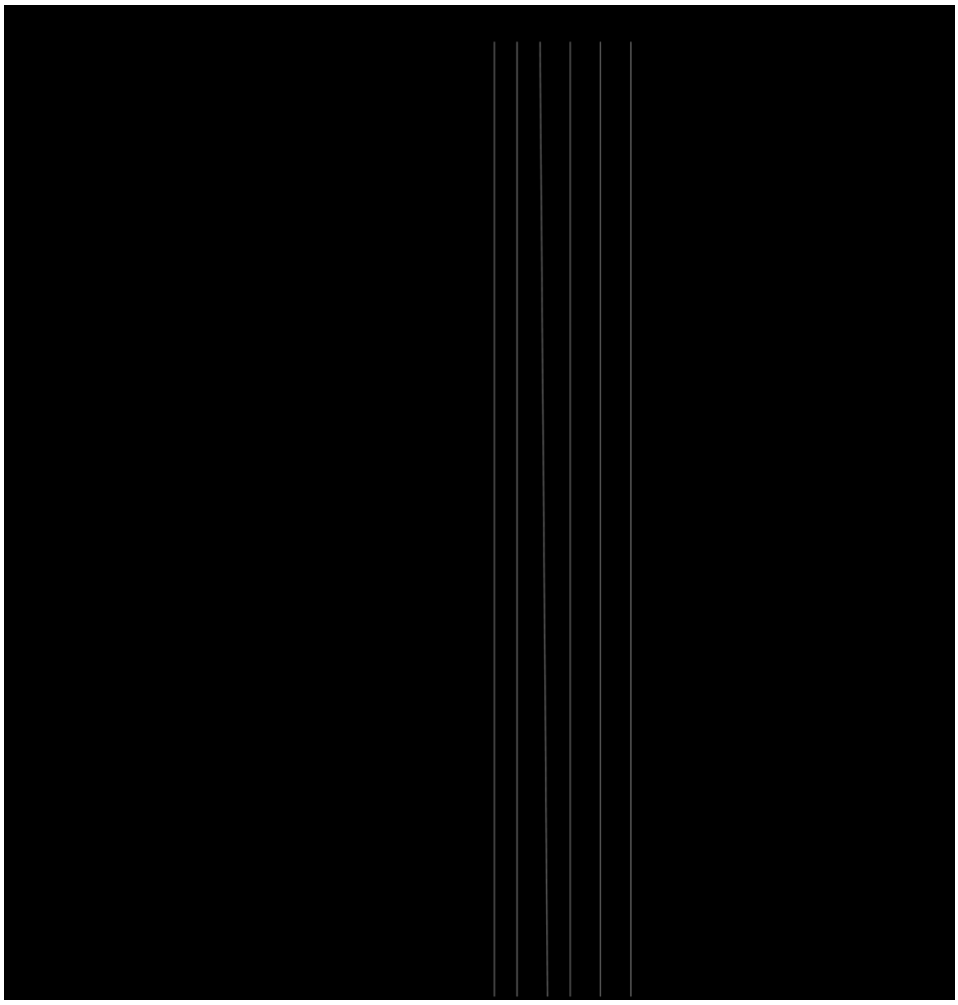
The deduced amino acid sequence of the isolated twelve individual antibody clones revealed insertions of up to eight amino acids in V_H CDR3 and frequent mutations in framework and CDR regions of V_L indicating that gene conversion and insertion of pseudogenes had taken place [167,169]. The CDR3 regions of both antibody chains showed the highest variability as was expected since the antigen binding region is built up to a large extent by these parts of the antibody. Amino acid substitutions within the constant framework regions flanking the variable CDRs provide an indication of somatic hypermutation involved in antibody gene diversification [223]. Framework mutations may affect binding affinities to a surprisingly large extent [224]. Five antibodies shared basically the same V_H chain but clearly differed in V_L segments. This so-called V_H promiscuity has been observed in human antibody fragments as well [225] and supports the notion that antibody heavy chains tend to have stronger contribution to antigen binding than light chains [226].

7.5 Antibody clone JM7_8 revealed a highly conserved H5 specific neutralizing epitope

Attempts to map the epitopes of two of the isolated antibodies concentrated on using a peptide spot membrane derived from the amino acid sequence of human H5

A/Anhui/1/2005. Mapping for the antibody clone JM7_8 revealed the six amino acid short epitope FRNVVW at amino acid positions 143-149 (H5 numbering [227] / H3 positions 147-153 H3 numbering [228]). This conserved linear structure is flanked by much more variable regions i) the antigenic region A [228] (RCSB file pdb 2hmg) also named 140 S loop (H5 positions 136-141), to which binding of neutralizing antibodies was already shown [229] and ii) the glycosylation site at position 158-160 [35]. Modelling revealed that the FRNVVW epitope is rather hidden in the structure of the hemagglutinin and deletion of the adjacent glycosylation site, as recently reported for emerging Asian H5 influenza viruses[230,231] could improve accessibility of the epitope and thus facilitate detection. The recently described N158D mutation confering improved replication to a droplet transmissible avian influenza virus in the ferret model[55] is close to but not within the defined conserved epitope. Since this mutation abolishes a glycosylation site its contribution to an improved accessiblity of the conserved epitope at position 148-153 can be assumed. Nevertheless, an influence of mutations at this glycosylation site on the shape and accessibility of the linear epitope remains to be investigated. Computational analyses have previously indicated that the defined epitope FRNVVW could be an antigenic peptide [227]. The tryptophane at the end of the epitope is involved in receptor binding, thus an antibody to this epitope can possibly have have neutralizing activity. Furthermore, from cross-protection vaccination studies a fifteen amino acid long epitope KSSFRNVVWLIKKN comprising the shorter six amino acid epitope was published and shown to be conserved among H5 influenza viruses and assumed to be involved in protection against heterologous H5 challenge[232]. Since the antibody JM7_8 directed against the short epitope FRNVVW was shown to bind a linear epitope, detected H5 from various influenza strains and demonstrated neutralization of at least H5 virus A/teal/Föhr/Wv632/05 cross-protection potential of this peptide sequence can be assumed. Sequence alignments of 2466 European,Asian, African, North American and Egyptian H5 virus isolates revealed a 95.7% conservation of the six amino acid epitope (Tab 16) (personal communication Abdelwhab, FLI, unpublished data). Thus, this epitope is highly conserved within H5 influenza viruses. Furthermore it is not present in H1 viruses.

Table 16: Comparison of hemagglutinin epitope conservation among H5 influenza virus isolates



Failure in neutralization of A/mallard/B.C./544/09 using the antibody JM7_8 may be due to glycosylation at position 158-160 which is typical of most American and old Asian H5 viruses[35] and may sterically hinder antibody binding. The same effect may have had led to negative results in AC-ELISA for this virus. This assumption is supported by the observation that this virus became detectable in AC-ELISA under mild denaturing conditions. Sterical hinderance may have been moderated under these conditions.

Up to now, no linear epitopes in the highly variable globular structure of influenza hemagglutinin Egyptian escape mutant A/chicken/Egypt/0879-NLQP/2008-R737/09 shared with other H5 influenza hemagglutinins were known.Thus, a highly conserved very short epitope applicable for vaccination studies and diagnostic purposes along with the respective neutralizing antibody has been elucidated.

Two different binding sites were revealed for antibody clone JM1_7. Epitope

ERRRKR of peptides 111-115 include the highly pathogenic cleavage site of the H5 hemagglutinin. The cleavage site of low pathogenic H5 influenza viruses consists of only one arginine. Since antibody clone JM7_1 has been shown to bind comparably well to H5 from highly and low pathogenic influenza viruses in IPMA and immunoblot assays, it is rather unlikely that this antibody provides specific binding of the influenza cleavage site region. The false positive signals on peptide spot membrane may be due unspecific binding to the positively charged arginines and lysines.The epitope VPKIATRSK (210–218 H5 numbering[227]) is a more likely correct epitope of antibody clone JM7_1 and has previously been predicted to be an antigenic peptide by computational methods[227]. This epitope is not as well conserved as the epitope of JM7_8 as JM7_1 did not detect the Egyptian escape mutant H5. Obviously this epitope is not restricted to H5 influenza viruses since the antibody clone JM7_1 showed cross-reactivity with H6.

Published data on further highly conserved epitopes in the head-like part of the H5 protein are rare. Chen et al have identified a neutralizing antibody binding a conformational epitope including positions 158 and 182[209,233] whereas Velumani and coworkers have described the antigenic linear epitope CNTQCNTP at positions 274-281 (H5 numbering[227])[234].

7.6 Possible applications in diagnostics, therapy and vaccination of isolated anti-H5-antibodies and the identified epitope

The antibodies isolated in this work can be used for diagnostic purposes in systems such as lateral flow devices (LFD), ELISA based applications and biosensors. Application of isolated antibodies in lateral flow devices can be proposed as highly specific broad H5 detecting antibodies have been isolated. As antibody clone JM7_8 was capable of detecting a large variety of H5 influenza viruses without showing cross-reactivity to heterosubtypic viruses this antibody has an outstanding potential for this application. Additionally, the determined subnanomolar affinities provide an excellent basis for the development of such a detection system, although it remains to be evaluated whether the achievable detection limits meet the requirements. Two commercially available LFDs (Anigen Rapid AIV Ag Test Kit, BioNote Inc., Korea; QuickVue Influenza test, QUIDEL, USA) are based on the detection of the viral nucleoprotein and are therefore not subtype specific. Both LFDs were tested in field situations in Egypt and revealed a detection limit of at least 10^4 virus particles[82,235] which is a 1000 fold lower sensitivity compared to PCR based detection. As up to

now only H5 and H7 influenza viruses have shown to be able to naturally convert from low pathogenic to highly pathogenic influenza viruses the proposed H5 subtype specific detection system would be of fundamental advantage for surveillance and disease control. Likewise, the isolated antibodies can be applied in biosensors. For H7 and H8 influenza virus detection the successful use of an inferometric biosensor has been reported[236]. As mentioned in section 6.2, Hong *et al*. have developed a magnetic biosensor capable of detecting the antigen in picomolar amounts, applicable for point-of-care diagnostics and in a 96-well format[216]. To support the use of biosensors in the field a miniaturised biosensor has been introduced[237].

ELISA based detection systems using two antibody entities binding the antigen may be difficult due to the need of complementary antibody pairs recognizing different epitopes and capable of detecting soluble hemagglutinin. With antibody clones JM7_1 and JM7-3 such an antibody pair could be proposed, yet not all analysed H5 viruses were detectable. Furthermore, broad range H5 detecting antibody JM7_8 could be used as capture antibody in ELISA based applications along with pooled hyperimmune sera for detection. The detection limit of 1 HAU in an AC-ELISA using anti-H5 and anti-N1 antibodies recognizing highly conserved epitopes has been reported [238]. A dot ELISA using complementary antibodies detecting highly conserved epitopes of H5 avian influenza viruses has been described[239].

Besides their usefulness in diagnostics, isolated antibodies may be used for therapeutic purposes with regard to passive immunization. In the case of a pandemic outbreak of influenza viruses neutralizing antibodies could be used for passive immunization and treatment.As long as no universal influenza antibody is available, subtype specific antibodies may be helpful. After the humanization of the isolated neutralizing antibody clones JM7_8 and JM7_3 by maintaining affinity, specificity and neutralizing activity the antibodies could be used in humans. A successfull humanization of chicken scFv antibodies has been demonstrated[240]. All isolated scFv antibodies were of chicken origin. An application of a complete scFv chicken antibody extented by a chicken Fc-part in passive poultry immunization could be possible. Yet economic cost benefit calculations clearly prevent this application. Given that the necessary antibody stability could be provided passive immunization of chickens via the oral route with egg yolk IgY is achievable. The successful oral immunization of chickens against *Vibrio cholerae* applying this method has been shown[241].

Most valuable result of this work with regard to vaccination is the elucidated highly specific linear epitope of antibody clone JM7_8. As this antibody shows neutralizing activity the respective linear epitope used as a vaccine potentially confers immune protection to H5 influenza viruses. The epitope is linear and short and therefore less prone to formation of a secondary structure that could destroy the epitope and hinder successful vaccination. Nevertheless, antigenicity of the epitope as well as vaccination conditions including adjuvants remain to be investigated. Wang et al. have shown a successful immunization in mice using a synthetic peptide mapping to the stalk-like part of the hemagglutinin. After immunization mice were protected against lethal challenge with influenza viruses of the subtypes H5, H3 and H1[242].

Additionally to these potential application of the already isolated antibodies it is noteworthy that constructed chicken antibody phage libraries most likely contain antibodies recognizing viral neuraminidase N1 which could easily be isolated and analysed for their characteristics, potentials and applications.

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11 Abbreviations

A	ampicillin
Abs 450 nm	Absorption at a wave lenght of 450 nm
AC-ELISA	Antigen-Capture-ELISA
AEC	3-amino-9-ethylcarbazole
AI	Avian Influenza
AIV	Avian Influenza Virus
AP	alkaline phosphatase
APS	ammonium peroxydedisulfate
ATV-D	
bp	basepairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumine
CBS	citrate buffered saline
CDR	Complementrity Determining Region
cfu	colony forming units
CIP	calf intestine protease
CH	Constant heavy (part of antibody heavy chain)
CL	Constant light (part of antibody light chain)
COO	Carboxylic (magnetic beads)
DAB	3,3' diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dYT	double yeast-tryptone
dNTP	deoxyribonucleoside triphosphate
ECE	embryonated chicken eggs
EC_{50}	half maximal effective concentration; here: used to express the half maximal absorption value in relation to antibody
EDC	ethanolamine hydrochloride
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EtOH amine	ethanolamine
f	forward
Fab	fragment antigen binding (part of antibody)
Fc	fragment crystallizable (part of antibody)
FCS	fetal calf serum
FDA	Food and Drug Administration

FLI	Friedrich-Loeffler-Institute/Federal Research Institute for Animal Health, Isle of Riems, Germany
Fv	fragment variable (part of antibody)
G	glucose
xg	gravity constant
HA	hemagglutinin
HA0	hemagglutinin precursor protein (uncleaved)
HA1	membrane distal part of the cleaved / activated hemagglutinin
HA2	membrane bound part of the cleaved / activated hemagglutinin
HA-titre	titre obtained in hemagglutination assay
HAU	hemagglutinating units
HI	Hemagglutination Inhibition Test
HAL	Human Antibody Library
HEK	Human Embryonic Kidney
His	histidin
HPAIV	highly pathogenic avian influenza virus
HRP	horseradish peroxidase
H5	influenza viruses of the hemagglutinin subtype H5
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IgA	immunoglobulin A
IgM	immunoglobulin M
IgY	chicken IgG-like immunoglobulin
IPMA	Immuno Peroxidase Monolayer Assay
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
kDa	kilodalton
KFC	Kentucky Fried Chicken (here: Name of Chicken Immune Library)
lac	lactose
P_{lacZ}	Lac promoter
log	logarithmic
LPAIV	low pathogenic avian influenza virus
LFD	Lateral flow device
MN	Macherey & Nagel
MPBST	milk powder (1 – 2 %) in PBS containing Tween 20 at final 0.05% (v/v)
MTP	Micro-titre plate

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	messenger-RNA
MW	molecular weight
NI	**N**euraminidase **I**nhibitors
negC	negative control
NBT	nitro-blue tetrazolium
NHS	N-hydroxysuccinimide
NS1	non-structural protein 1; influenza virus protein
OD_{600}	optical density at λ=600 nm
OIE	Office Internationale des Epizooties (World Organization for Animal Health)
ori	origin of replication
ORF	open reading frame
p.a.	per analysis
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline including Tween 20 final 0.05%
PCR	polymerase chain reaction
PDB	phage dilution buffer
pelB	Sequence of a signalling peptide for protein segregation into periplasm
pET	plasmid for protein expression using T7 RNA polymerase
PMSF	phenylmethanesulfonylfluoride
PP	polypropylene
PVDF	polyvinylidene fluoride
r	reverse
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
SA	streptavidin
scFv	single-chain variable fragment (part of antibody)
scFv-hIgG1Fc	single-chain antibody fused to the Fc-domain of human IgG1
scFv-mIgG1Fc	single-chain antibody fused to the Fc-domain of mouse IgG1
scFv-mIgG2cFv	single-chain antibody fused to the Fc-domain of mouse IgG2c
SDS	sodium dodecyl sulfate
SOC	Super Optimal broth with Catabolic repression
SOIV	swine origin influenza virus H1N1 (influenza pandemic 2009)
T	tetracycline

TAE	tris-acetate-EDTA
TBS	tris-buffered saline
$TCID_{50}$	
TEMED	N,N,N',N'-tetramethyl- ethane-1,2-diamine
Tris	Tris-(hydroxymethyl)-aminomethane
TMB	3,3′,5,5′-Tetramethylbenzidine
U	Unit
oN	over night
UV	ultraviolet
VH	variable heavy (part of antibody heavy chain)
VL	variable light (part of antibody light chain)
VN	virus neutralization
vRNP	viral ribonucleoprotein
vRdRp	viral RNA-dependent RNA polymerase
WB	Western Blot (immuoblot)
(w/v)	weight per volume

12 Appendix

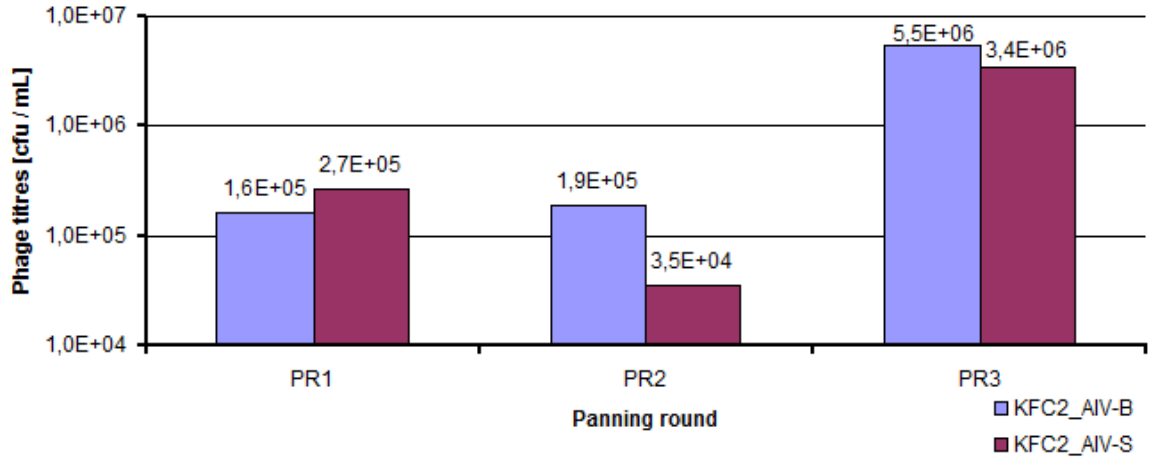

Figure 37: Phage titres after pannings on human H5 immobilized on carboxy-beads using chicken immune anti-H5 libraries (PR1-3: the three successive rounds of pannings).

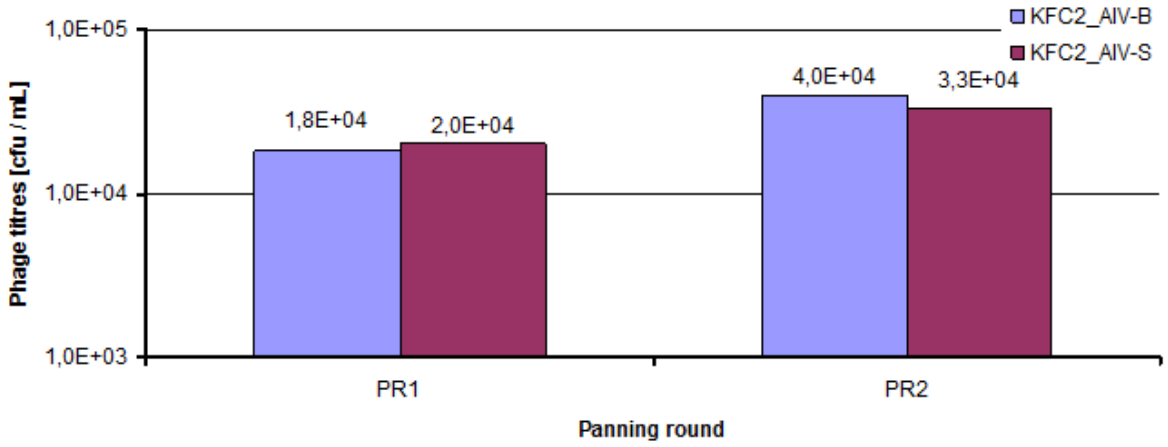

Figure 38: Phage titres a after pannings on avian H5 immobilized on Streptavidin beads using chicken immune anti-H5 libraries

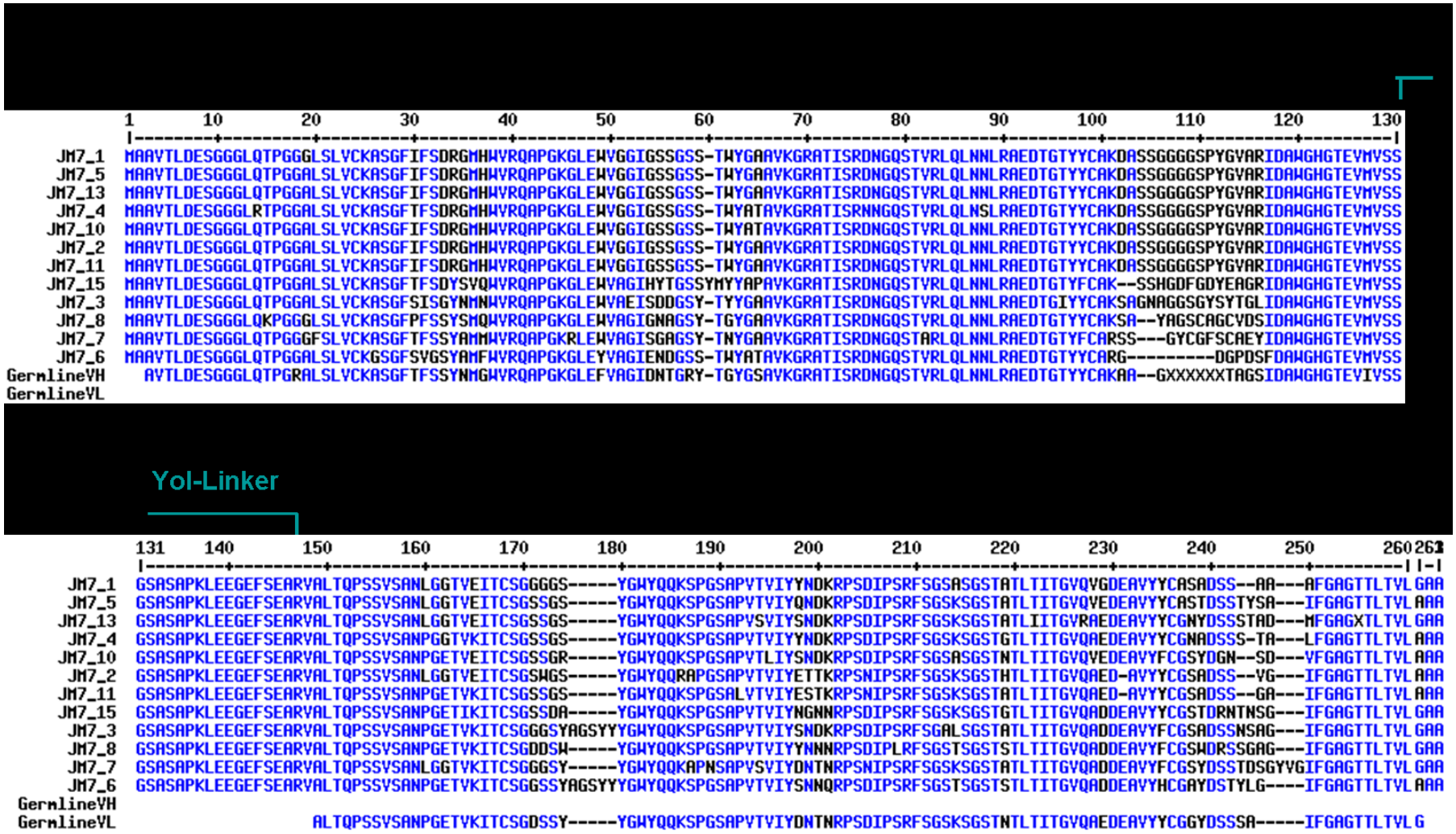

Figure 39: Amino acid sequence alignment of isolated anti-H5 chicken antibodies.
Amino acid identity to the germ-line sequences are shown in grey (cyan), differences in black, absence of corresponding residue by dashes. CDR and Yol-linker of scFv annotated. As germline reference sequences AAA48861.1 (NCBI database) was used for VL and the VH sequence is according to Yamanaka (1996) with an X indicating hypervariable positions.

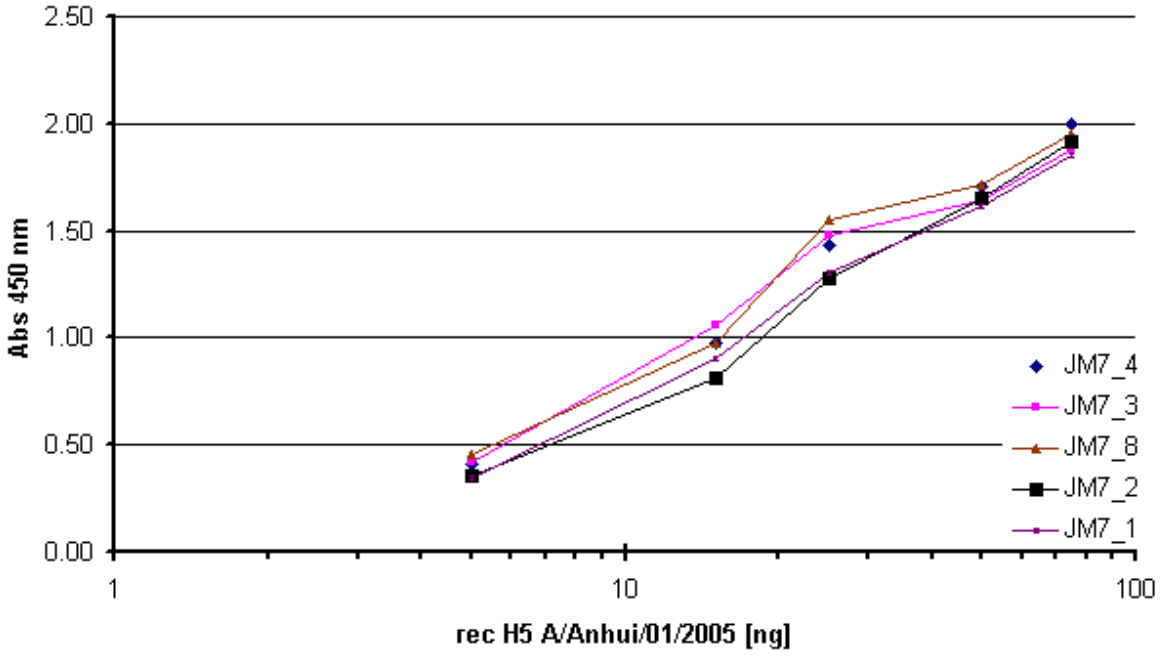

Figure 40: Titration ELISA of human H5 A/Anhui/1/2005 using scFv-hIgG1Fc antibodies.
H5 was immobilized to ELISA plates in a range between 5 – 75 ng/well and detected using 500ng scFv-hIgG1Fc antibodies per well and HRP-labeled goat-anti-human-Fc-antibody (Sigma A0170).

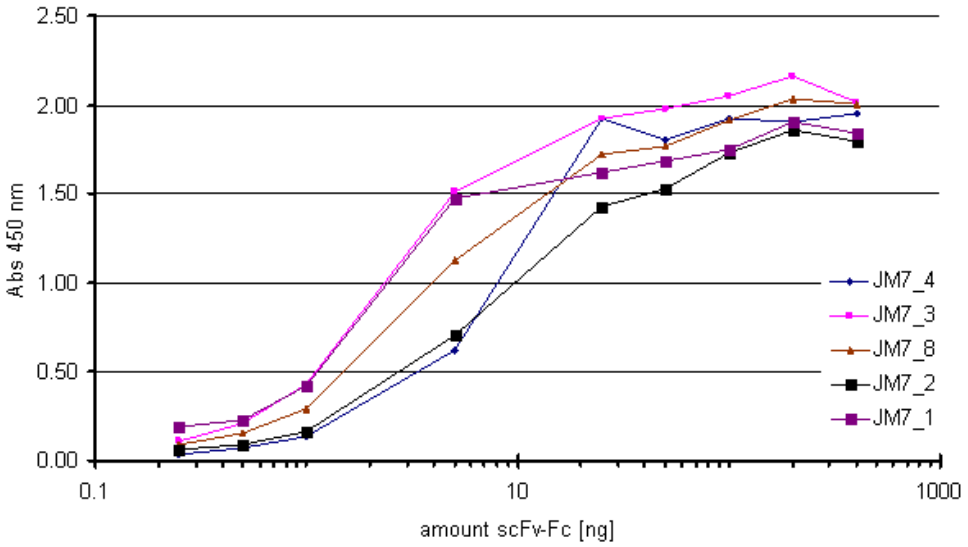

Figure 41: Titration of scFv-hIgG1Fc antibodies on human H5 A/Anhui/1/2005.
50 ng of recombinant H5 A/Anhui/1/2005 was immobilized to ELISA plates.
Antibody binding was analysed in a range of 400-0.25 ng/ well and target bound antibodies were detected using HRP-labeled goat-anti-human-Fc-antibody (Sigma A0170).

Epitope membrane peptides using hemagglutinin of H5N1 A/Anhui/1/2005

1	MEKIVLLLAIVSLVK
2	IVLLLAIVSLVKSDQ
3	LLAIVSLVKSDQICI
4	IVSLVKSDQICIGYH
5	LVKSDQICIGYHANN
6	SDQICIGYHANNSTE
7	ICIGYHANNSTEQVD
8	GYHANNSTEQVDTIM
9	ANNSTEQVDTIMEKN
10	STEQVDTIMEKNVTV
11	QVDTIMEKNVTVTHA
12	TIMEKNVTVTHAQDI
13	EKNVTVTHAQDILEK
14	VTVTHAQDILEKTHN
15	THAQDILEKTHNGKL
16	QDILEKTHNGKLCDL
17	LEKTHNGKLCDLDGV
18	THNGKLCDLDGVKPL
19	GKLCDLDGVKPLILR
20	CDLDGVKPLILRDCS
21	DGVKPLILRDCSVAG
22	KPLILRDCSVAGWLL
23	ILRDCSVAGWLLGNP
24	DCSVAGWLLGNPMCD
25	VAGWLLGNPMCDEFI
26	WLLGNPMCDEFINVP
27	GNPMCDEFINVPEWS
28	MCDEFINVPEWSYIV
29	EFINVPEWSYIVEKA
30	NVPEWSYIVEKANPA
31	EWSYIVEKANPANDL
32	YIVEKANPANDLCYP
33	EKANPANDLCYPGNF
34	NPANDLCYPGNFNDY
35	NDLCYPGNFNDYEEL
36	CYPGNFNDYEELKHL
37	GNFNDYEELKHLLSR
38	NDYEELKHLLSRINH
39	EELKHLLSRINHFEK
40	KHLLSRINHFEKIQI
41	LSRINHFEKIQIIPK
42	INHFEKIQIIPKSSW
43	FEKIQIIPKSSWSDH
44	IQIIPKSSWSDHEAS
45	IPKSSWSDHEASSGV
46	SSWSDHEASSGVSSA
47	SDHEASSGVSSACPY
48	EASSGVSSACPYQGT
49	SGVSSACPYQGTPSF
50	SSACPYQGTPSFFRN
51	CPYQGTPSFFRNVVW
52	QGTPSFFRNVVWLIK
53	PSFFRNVVWLIKKNN
54	FRNVVWLIKKNNTYP
55	VVWLIKKNNTYPTIK
56	LIKKNNTYPTIKRSY
57	KNNTYPTIKRSYNNT
58	TYPTIKRSYNNTNQE
59	TIKRSYNNTNQEDLL
60	RSYNNTNQEDLLILW
61	NNTNQEDLLILWGIH
62	NQEDLLILWGIHHSN
63	DLLILWGIHHSNDAA
64	ILWGIHHSNDAAEQT
65	GIHHSNDAAEQTKLY
66	HSNDAAEQTKLYQNP
67	DAAEQTKLYQNPTTY
68	EQTKLYQNPTTYISV
69	KLYQNPTTYISVGTS
70	QNPTTYISVGTSTLN
71	TTYISVGTSTLNQRL
72	ISVGTSTLNQRLVPK
73	GTSTLNQRLVPKIAT
74	TLNQRLVPKIATRSK
75	QRLVPKIATRSKVNG
76	VPKIATRSKVNGQSG
77	IATRSKVNGQSGRMD
78	RSKVNGQSGRMDFFW
79	VNGQSGRMDFFWTIL
80	QSGRMDFFWTILKPN
81	RMDFFWTILKPNDAI
82	FFWTILKPNDAINFE
83	TILKPNDAINFESNG
84	KPNDAINFESNGNFI
85	DAINFESNGNFIAPE
86	NFESNGNFIAPEYAY
87	SNGNFIAPEYAYKIV
88	NFIAPEYAYKIVKKG
89	APEYAYKIVKKGDSA
90	YAYKIVKKGDSAIVK
91	KIVKKGDSAIVKSEV
92	KKGDSAIVKSEVEYG
93	DSAIVKSEVEYGNCN
94	IVKSEVEYGNCNTKC
95	SEVEYGNCNTKCQTP
96	EYGNCNTKCQTPIGA
97	NCNTKCQTPIGAINS
98	TKCQTPIGAINSSMP
99	QTPIGAINSSMPFHN
100	IGAINSSMPFHNIHP

101 INSSMPFHNIHPLTI
102 SMPFHNIHPLTIGEC
103 FHNIHPLTIGECPKY
104 IHPLTIGECPKYVKS
105 LTIGECPKYVKSNKL
106 GECPKYVKSNKLVLA
107 PKYVKSNKLVLATGL
108 VKSNKLVLATGLRNS
109 NKLVLATGLRNSPLR
110 VLATGLRNSPLRERR
111 TGLRNSPLRERRRKR
112 RNSPLRERRRKRGLF
113 PLRERRRKRGLFGAI
114 ERRRKRGLFGAIAGF
115 RKRGLFGAIAGFIEG
116 GLFGAIAGFIEGGWQ
117 GAIAGFIEGGWQGMV
118 AGFIEGGWQGMVDGW
119 IEGGWQGMVDGWYGY
120 GWQGMVDGWYGYHHS
121 GMVDGWYGYHHSNEQ
122 DGWYGYHHSNEQGSG
123 YGYHHSNEQGSGYAA
124 HHSNEQGSGYAADKE
125 NEQGSGYAADKESTQ
126 GSGYAADKESTQKAI
127 YAADKESTQKAIDGV
128 DKESTQKAIDGVTNK
129 STQKAIDGVTNKVNS
130 KAIDGVTNKVNSIID
131 DGVTNKVNSIIDKMN
132 TNKVNSIIDKMNTQF
133 VNSIIDKMNTQFEAV
134 IIDKMNTQFEAVGRE
135 KMNTQFEAVGREFNN
136 TQFEAVGREFNNLER
137 EAVGREFNNLERRIE
138 GREFNNLERRIENLN
139 FNNLERRIENLNKKM
140 LERRIENLNKKMEDG
141 RIENLNKKMEDGFLD
142 NLNKKMEDGFLDVWT
143 KKMEDGFLDVWTYNA
144 EDGFLDVWTYNAELL
145 FLDVWTYNAELLVLM
146 VWTYNAELLVLMENE
147 YNAELLVLMENERTL
148 ELLVLMENERTLDFH
149 VLMENERTLDFHDSN
150 ENERTLDFHDSNVKN
151 RTLDFHDSNVKNLYD
152 DFHDSNVKNLYDKVR
153 DSNVKNLYDKVRLQL
154 VKNLYDKVRLQLRDN
155 LYDKVRLQLRDNAKE
156 KVRLQLRDNAKELGN
157 LQLRDNAKELGNGCF
158 RDNAKELGNGCFEFY
159 AKELGNGCFEFYHKC
160 LGNGCFEFYHKCDNE
161 GCFEFYHKCDNECME
162 EFYHKCDNECMESVR
163 HKCDNECMESVRNGT
164 DNECMESVRNGTYDY
165 CMESVRNGTYDYPQY
166 SVRNGTYDYPQYSEE
167 NGTYDYPQYSEEARL
168 YDYPQYSEEARLKRE
169 PQYSEEARLKREEIS
170 SEEARLKREEISGVK
171 ARLKREEISGVKLES
172 KREEISGVKLESIGT
173 EISGVKLESIGTYQI
174 GVKLESIGTYQILSI
175 LESIGTYQILSIYST
176 IGTYQILSIYSTVAS
177 YQILSIYSTVASSLA
178 LSIYSTVASSLALAI
179 YSTVASSLALAIMVA
180 VASSLALAIMVAGLS
181 SLALAIMVAGLSLWM
182 LAIMVAGLSLWMCSN
183 MVAGLSLWMCSNGSL
184 GLSLWMCSNGSLQCR
185 LWMCSNGSLQCRICI

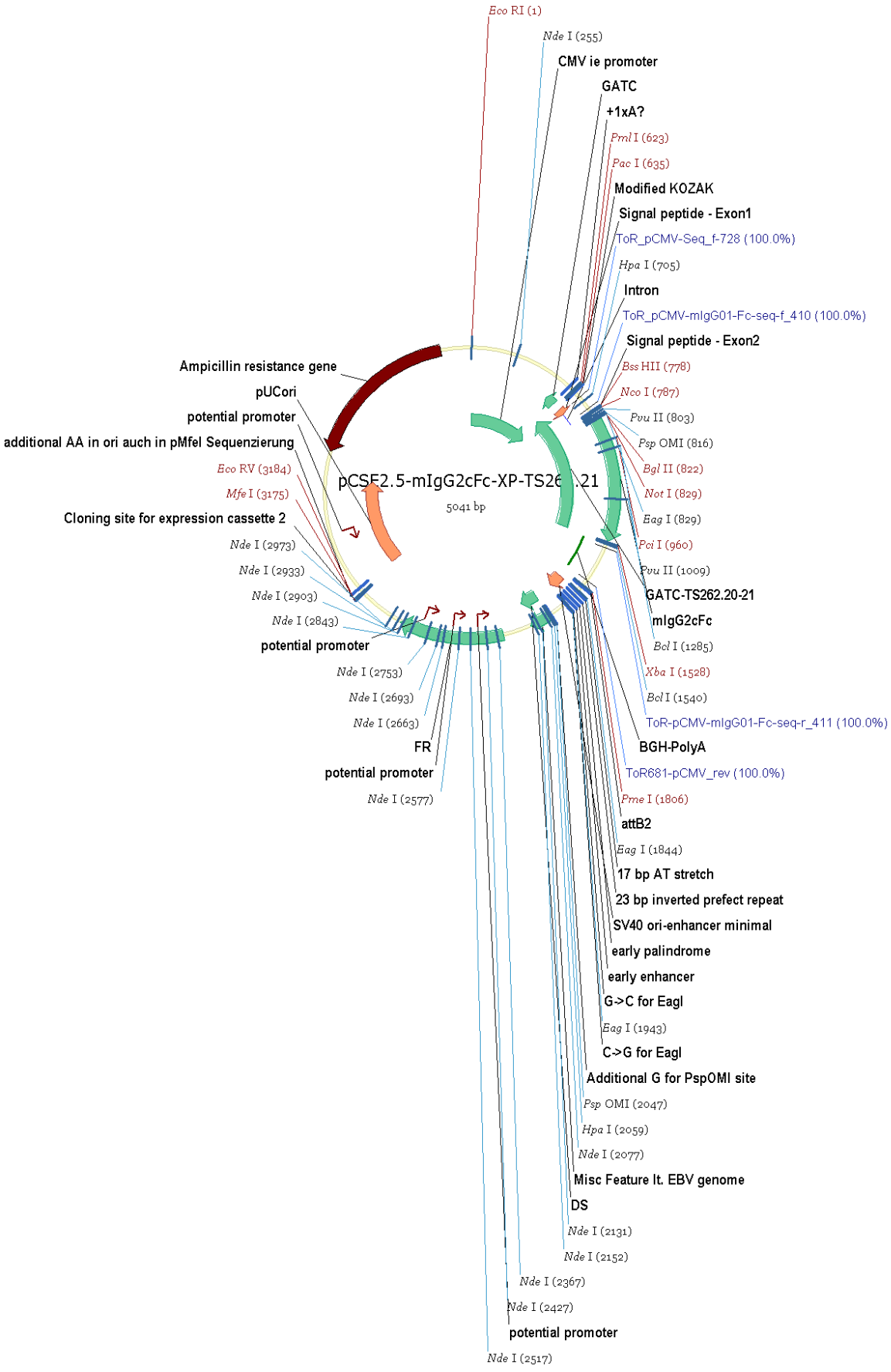

Figure 42: Plasmid map for pCSE2.5-mIgG2cFc-XP-TS262.21 vector allowing the expression of scFv-mIgG2cFc antibodies in eukaryotic cells.

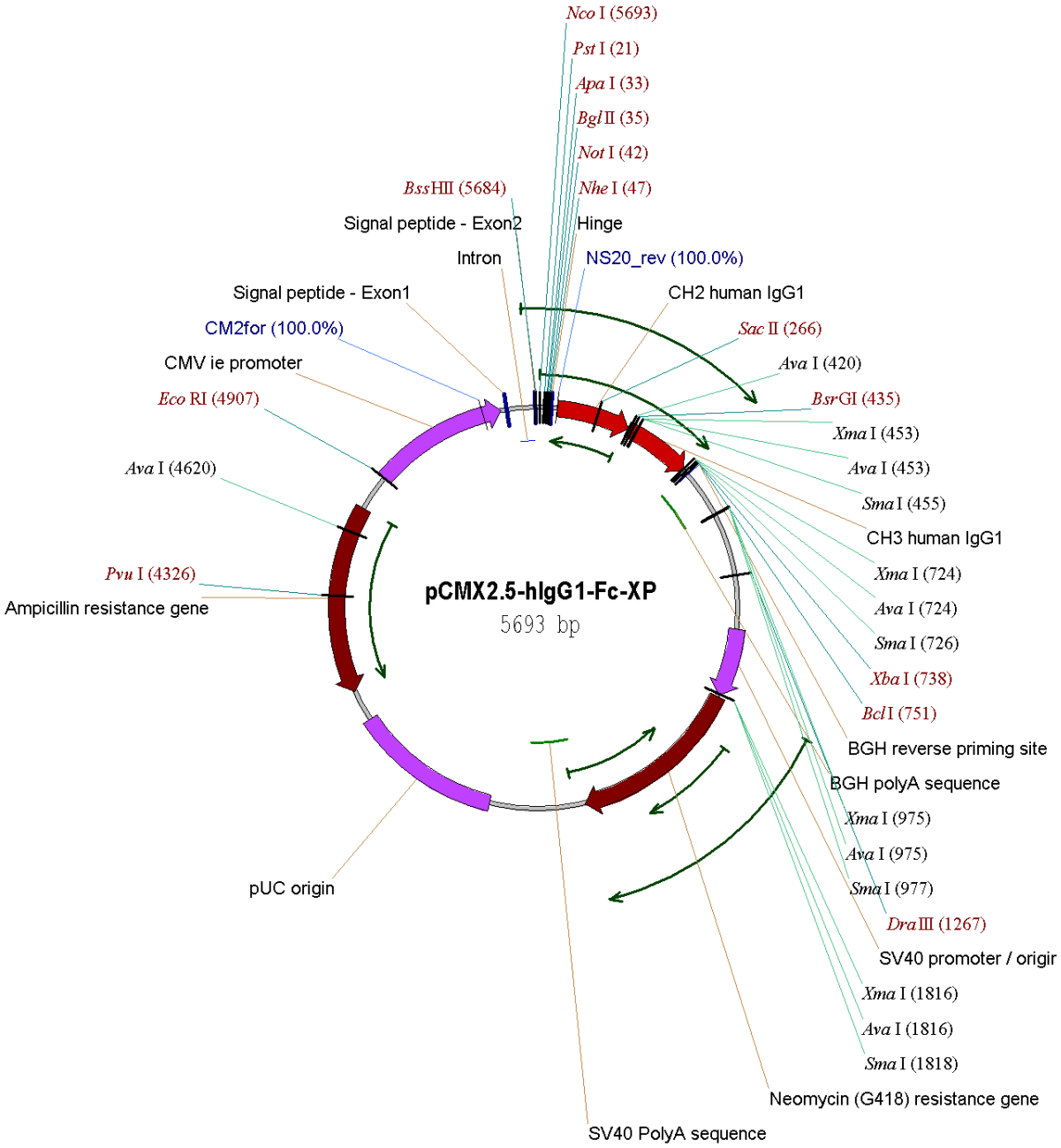

Figure 43: Plasmid map for pCMX2.5-hIgG1Fc-XP vector allowing the expression of scFv-hIgG1Fc antibodies in eukaryotic cells.

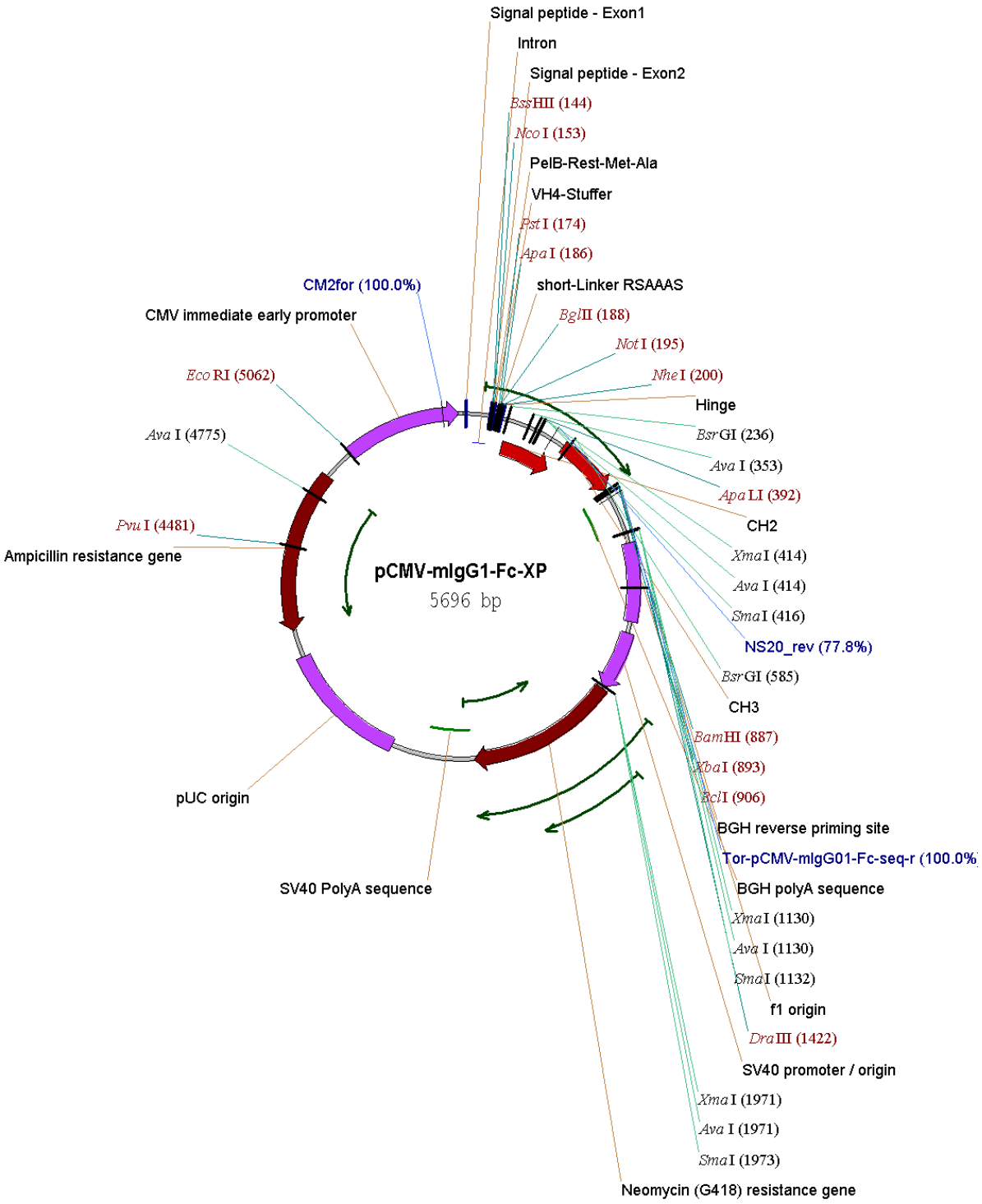

Figure 44: Plasmid map for pCMV-mIgG1Fc-XP vector allowing the expression of scFv-mIgG1Fc antibodies in eukaryotic cells.

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